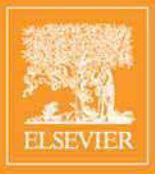

ADVANCES IN CANCER RESEARCH

Volume II

Jesse. P. Greenstein &
Alexander Haddow

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ADVANCES IN CANCER RESEARCH

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Volume II

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PREFACE

In the year which has elapsed since the publication of Volume I of *Advances in Cancer Research*, the editors have been gratified by the favorable reception accorded to it, equally by their fellow workers and by the scientific press. This they regard not only as a general encouragement of the new enterprise but also as a reflection of the value, certainly no less in this than in any other scientific field, of the authoritative review. Since their aim is not only to consolidate the venture but, so far as is possible, to improve it, they take the present opportunity of welcoming suggestions to this end, whether concerned with particular topics or aspects which most merit inclusion, or with policy as a whole.

In the current volume, the central theme of carcinogenesis again finds a prominent place, as, for example, in the review of carcinogenesis and tumor pathogenesis by Dr. I. Berenblum, whose own work has elucidated so many of the factors involved; and in that of Dr. G. M. Badger, who, not only as a pupil of Professor J. W. Cook at the Royal Cancer Hospital in London and at the University of Glasgow, but also by virtue of his own contributions subsequently, is especially qualified to deal with the relationships of chemical constitution and carcinogenic activity. Although the list of chemical carcinogens is doubtless still incomplete, and much may still be learned from the chemical interrelationships of those already known, within the past few years a new emphasis has inevitably been given to problems of their mode of action, involving a shift of interest toward those macromolecular receptors with which, it would appear, they most probably combine. This development has been greatly stimulated by the discovery of carcinogenic activity in a whole range of "biological alkylating agents," the reactions of which, among other carcinogens, are dealt with in a contribution by Dr. P. Alexander. So far at least as their biological end-results are concerned, many of these alkylating agents may very reasonably be described as "radiomimetic," lending special significance to the facts, first that the earliest experimentally induced tumors owed their origin to ionizing radiations, and, second, that the total of carcinogenic agents has been greatly increased, through the wealth of radioisotopes accruing from the atomic energy programs of the past ten years; these, and their practical bearings, are considered in a contribution by Dr. A. M. Brues.

From its beginnings, research into the etiology of cancer has been

enlivened by the controversy between those who subscribe to the importance of a process of infection—especially by viral agents—and those others who believe the reverse. Even though many must judge the antithesis to be a false one, the issue in reality is still undecided; contributions which throw light upon it include a vigorous review by Dr. C. Oberling and Dr. M. Guérin, a survey of genetic studies by Dr. L. W. Law, and an account, by Dr. J. Craigie, of the survival and preservation of tumors in the frozen state—the last including details not merely of great theoretical interest but of much practical value as well.

Apart altogether from etiology and pathogenesis, increased note has been paid of recent years to the systemic and nutritional consequences of tumor growth once established, and the review by Dr. L. D. Fenninger and Dr. G. Burroughs Mider, on energy and nitrogen metabolism, is of special interest for its relevance to the disease in man. The same applies in marked degree to the contribution of Dr. C. Chester Stock on experimental cancer chemotherapy, and that of Dr. C. T. Klopp and Dr. Jeanne C. Bateman on the clinical use of the nitrogen mustards. Admitted that the chemotherapeutic agents of the future are likely to be very different in their nature, specificity, and potency from those at present used, it is a matter of no little consequence that limited gains continue to be recorded, and that the harvest of benefit grows, even if slowly, from year to year.

It is our ambition, and hope, that these volumes may serve not merely as an annual chronicle of progress, but as a recurring stimulus to the work ahead.

THE EDITORS

February, 1954

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The Reactions of Carcinogens with Macromolecules

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I. Introduction

The ever growing list of carcinogenic agents (cf. Hartwell, 1951; Haddow, 1953) reveals that the property of inducing cancer in experimental animals is shared by such a wide variety of chemical substances and physical agents as to render it almost impossible to conceive of a common chemical mechanism. Moreover, the possibility that the causative agent may be a metabolic product has made any comparison of the chemical reactivities of the carcinogens of doubtful value in connection with the problem of their mode of action. Nevertheless, in view of our limited knowledge of the processes underlying cell division, a promising approach is the search for a common end effect, which can be brought about by different chemical reactions such as the modification of a macromolecule.

Although the polycyclic hydrocarbons, azo compounds and physical agents have been investigated most thoroughly and for the longest period, the more recent recognition of carcinogenicity in a whole series of biological alkylating agents, the sulfur and nitrogen mustards, epoxides, ethyleneimines, and dimesyloxyalkanes, has greatly advanced our understanding. These substances, varying widely in chemical composition and having no common physical properties, share only on the ability to alkylate certain groupings (see p. 6). Since any metabolic reaction is almost certain to bring about the loss of this chemical reactivity, it can be concluded with some confidence that it is the original substances which bring about the initial chemical changes which lead to carcinogenicity; also since the overall configuration of these molecules can be widely varied it appears unlikely that a highly specific adsorption process, always difficult to study experimentally, plays a part in their mechanism of action, which can therefore be tentatively ascribed to a definite chemical reaction. For these reasons the carcinogenic alkylating agents will be considered first in this review.

The study of the reaction of carcinogenic agents with macromolecules has received a great impetus from current genetical studies, which strongly indicate that their site of action is the nucleus and that changes produced in the cytoplasm are of secondary importance. Moreover, convincing evidence points to the conclusion that a direct reaction with the chromosomes occurs and that this possibility leads to a mutation by loss resulting in the failure to produce a regulating enzyme (Haddow, 1953). Marked variations in the changes produced in the cell nucleus have been observed with different carcinogens (Koller and Casarini, 1952)

without, however, invalidating the general picture that they react with the chromosomes directly or with macromolecules prior to their incorporation into the chromosome. Although the enzymology of the carcinogens is beyond the scope of this review, it may be relevant to point out in support of the hypothesis outlined above that no enzyme system has so far been found which is powerfully inhibited by all the carcinogenic alkylating agents. For instance, Adams and Thompson (1948) found nitrogen mustard a selective inhibitor of cholinesterase, but Bullock (1952) showed that this property is not shared by the epoxides or mesyloxyalkanes. Boyland *et al.* (1951) found an exactly similar position for the enzyme hexokinase.

Little is known about the chemical composition or the macromolecular structure of the chromosomes except that they consist essentially of deoxyribosenucleic acid and proteins. There are essentially two points of view concerning their relative functions; the first (cf. Haurowitz, 1950) suggests that the biological specificity resides in the protein moiety and that the nucleic acid serves to maintain the protein in an expanded configuration necessary for its function as a "template."

Alternatively, it has been proposed (cf. Stern, 1949) that the "gene codes" are incorporated in the nucleic acid chains which are protected by the proteins. The latter view receives direct support from the finding that directed mutations can be produced in bacteria by introducing pure deoxyribose nucleic acids which show a very high degree of specificity (Ephrussi-Taylor, 1951; Avery, 1944; Boivin, 1947). A possible interpretation of these experiments is that certain genes in these microorganisms can be identified with specific nucleic acid. A similar conclusion can be reached in the case of fish from the work of Felix *et al.* (1951) who showed that the sperm heads of river trout after careful removal of cytoplasm by plasmolysis consists entirely of a nucleoprotein made up of deoxyribonucleic acid and clupein. These results were confirmed by the reviewer, who found that 96% of the contents of the sperm heads of herring could be accounted for as protamine and nucleic acid; the deviation from 100% is not significant and is within experimental error. These results are in direct opposition to those of E. and E. Stedman who believe that another protein is present. To eliminate the possibility that nuclear material is lost during the isolation Felix *et al.* (1952) examined the viability of the sperm heads so obtained and demonstrated that these could still fertilize an egg. The protamines are not homogeneous (Rasmussen, 1934; Daimler, 1952) and are proteins of very low molecular weight containing only six or possibly seven different amino acids (cf. Linderstrom-Lang, 1933). Although the theoretically possible number of variants is still large it seems unlikely that so simple a molecule can act as gene codes, and it

appears likely that the genetically specific materials in fish sperm are nucleic acids.

These considerations indicate that the recent emphasis on the reaction of carcinogens with deoxyribonucleic acids is well founded. Since our understanding of the structure of these macromolecules, in particular their size and shape, is as yet very incomplete and also since nucleic acids contain many different reactive centers, which cannot always be easily distinguished, experiments with model substances, simulating one or other of the properties of nucleic acids, are relevant and may materially aid in the understanding of the biochemical reactions. The speculative nature of the chromosome-poison hypothesis discussed above and its corollary, the importance of nucleic acids, does not of course justify a neglect of the reactions of carcinogens with proteins. This is illustrated by the very important results which have been obtained from the study of the combination *in vivo* of the amino azo benzenes with proteins (see p. 63).

II. Biological Alkylating Agents

The organic chemistry and general reactions of the biologically active alkylating agents has already been reviewed in detail by W. C. J. Ross in Vol. I of this series. In the present article emphasis is placed on the reactions of these substances with macromolecules. The first part of this section deals to some extent with the field covered by Dr. Ross. This has been done deliberately because considerable progress has been made since 1950 when Dr. Ross's article was written, and because the new data are often relevant to the thesis of this review and cannot be presented in isolation.

1. Criteria for Biological Activity

Following on the discovery of the cytotoxic and growth inhibitory properties of the so-called sulfur and nitrogen "mustards" concerning which an extensive literature has accumulated which has been excellently reviewed by Philips (1950), considerations of possible mechanisms and chemical reactivity of these substances led to the simultaneous discovery by a group of workers at the Royal Cancer Hospital (cf. Haddow, 1951; Everett and Kon, 1950; Ross, 1950) and at the laboratories of the Imperial Chemical Industries Ltd. (Hendry, Rose, and Walpole, 1950; Hendry *et al.*, 1951b,c) of similar biological properties to those of the mustards in a series of bis-epoxides and ethyleneimines. The growth-inhibiting properties of polyfunctional ethyleneimines was also recognized at about the same time at the Sloan-Kettering Institute, New York (Philips and Thiersch, 1950). Progressive modifications in the chemical

constitution of the nitrogen "mustard" molecule led Timmis (1948) to another type of alkylating agent, the methanesulfonic acid esters of polyalcohols which were again found to possess biological activity similar to that of the mustards (Haddow and Timmis, 1950, 1951). In view of the close relationship between growth inhibitory activity and tumor induction, which had been found by Haddow (1935) and which had led to the discovery of carcinogenicity in the amino stilbenes (Haddow *et al.*, 1948) representative compounds from these different series were tested for this activity. Abundant evidence for the carcinogenicity of these compounds was found by Haddow and others (summarized by Haddow, 1953) and compounds containing the following groups, as well as satisfying a number of other conditions which will be discussed below, must now be considered as chemical carcinogens:

$-S\cdot CH_2CH_2Cl$	Sulfur "mustard"
$>N\cdot CH_2CH_2Cl$	Nitrogen "mustards"
$-CH(O)CH_2$	Epoxides
$CH_2(N)CH_2$	Ethylene imines
CH_3SO_2O-	Methanesulfonates

Since many more compounds have been tested for growth inhibition than for tumor induction and also since the close parallelism between these two types of activity is particularly impressive with these compounds (Haddow, 1953), results from growth inhibition tests will be used in the discussion of the factors which influence the carcinogenicity of these compounds. They have all been referred to as "radiomimetic poisons," since in their biological effects they closely simulate ionic radiations (Dustin, 1947; Boyland, 1952a), and Boyland (1948) had shown that in mice a dose of 1 mg./kg. of nitrogen mustard was equivalent to a total body irradiation of 300 to 400 r. This apparent correlation appeared to be further strengthened in that nitrogen mustard and x-rays both brought about a decrease in the viscosity of deoxyribose nucleic acid but more detailed investigation of both the *in vitro* (see p. 25) and the *in vivo* effects has shown that the similarities are more apparent than real; thus Koller and Casarini (1952) reached the conclusion, from a detailed cytological investigation, that "it would be a gross error to infer a similarity of the mode of action of nitrogen mustard and x-rays, based on the similarity of some 'end-products' (e.g., blistering of skin, bleaching of pigmented hair, etc.). The latter are the result of a complex

chain of reactions, which can be initiated by fundamentally different primary events. We believe this to be the case, and for that reason the objection is raised to labelling nitrogen mustard as a radiomimetic poison. Such an adjective, by implicitly emphasizing the resemblance of a series of biological phenomena which are end-products, tends to obscure the important basic differences." Abundant evidence supporting the view of Koller and Casarini (1952) will be found in the reactions of these agents with macromolecules. The reviewer feels, however, that the term "radiomimetic" can usefully be retained since it concisely summarizes the biological activity of these substances.

A. *Nature of Chemical Reactivity.* The one feature which all these substances have in common is their ability to act as alkylating agents under physiological conditions. The potential importance of such a reaction in connection with the biological activity of sulfur "mustard" was first emphasized by Peters in 1947. A further characteristic of all these compounds (cf. Ross, 1953) is that they are electrophilic and most readily react with so-called nucleophilic (i.e., electron rich) groups with the result that they will alkylate amines anions of organic and inorganic acids and atoms carrying a lone pair of electrons such as nitrogen, sulfur, or phosphorus. These alkylating agents react much more slowly with the above-mentioned groups when rendered less nucleophilic by the addition of a proton. The position may be summarized in the following way:

Groupings	Reactive Form	Less-reactive Form
Organic acid	$R{\cdot}COO^-$	$R{\cdot}COOH$
Hydroxy or phenolic compound	$R{\cdot}O^-$	$R{\cdot}OH$
Sulfydryl	$R{\cdot}S{\cdot}^-$	$R{\cdot}SH$
Amine	R_3N	$R_3\overset{+}{N}H$
Thio ether	$R{\cdot}S{\cdot}R{\cdot}$	—
Phosphorus compounds	PR_3 (trivalent)	OPR_3 (pentavalent)

It should be stressed, however, that even in their reactive forms the various groupings show very pronounced differences in their reactivity toward the electrophilic center which is characterized by a so-called competition factor (see p. 12). In the case of both the sulfur and nitrogen mustards the electrophilic character arises from a dissociation into an ethylene-immonium (or sulfonium) ion or alternatively into a carbonium ion, thus:

$$R_2N{\cdot}CH_2CH_2Cl \rightarrow R_2N{\cdot}CH_2CH_2^+ + Cl^-$$

This reaction will also occur if the halogen is replaced by another group of high electronegativity. Haddow and Timmis (1951) examined a series

of substituents which might resemble the chlorine atoms in the nitrogen mustards and found sulfonic acid esters, $—OSO_2R$, active but phosphoric acid esters, $—OPO(OR)_2$, and sulfuric acid esters, $—OSO_3R$, inactive. In the case of epoxides, ethyleneimines, and mesyloxy compounds the electrophilic character does not arise from an ionic dissociation but is the result of the displacement of one nucleophilic group (e.g., the methanesulfonate ion) by another (e.g., carboxyl) in a bimolecular (S_N2) process:

$$R{\cdot}OSO_2CH_3 + R'COO^- \rightarrow R{\cdot}OOCR' + OSO_2CH_3^-$$

The mechanism for the epoxides and ethylene imines though more complex is fundamentally similar. Direct support for this view is found in the fact that in general active ethylene imine derivatives are only formed by substitution at the NH group which will lead to a decrease in its basicity. The physicochemical evidence for this reaction mechanism has been summarized by Ross (1953) and references to the original publication will not be given here.

One of the most important consequences of this mechanism is that the radiomimetic alkylating agents will be unreactive towards an un-ionized —SH groups, although very reactive towards an ionized $—S^-$ groups which have in fact a higher competition factor (see p. 12) than any other anion likely to be present in a biological system. A satisfactory explanation can be given for the apparent anomaly that mustard gas, though reacting readily with single thiols, does not in general inactivate —SH enzymes *in vivo* (cf. Peters, 1947; Needham, 1948). This is in all probability due to the fact that the SH group in protein has a dissociation constant of about 10 and under physiological conditions less than 0.1% would be in the reactive form. Whereas the highly reactive thiols (cf. Ogston, 1948) are all appreciably dissociated at pH 7. There is considerable amount of evidence that the presence of alkylating groups which are not electrophilic (i.e., those which react readily with —SH groups and un-ionized organic acids) does not confer radiomimetic activity on a compound even if all the other criteria (see below) for activity are fulfilled. For example, compounds which are characterized by high reactivity to amino (and also SH) groups such as isocyanates, halogeno pyrimidines (Hendry *et al.*, 1951a), and halogeno-2,4-dinitrobenzene are also inactive; thus hexamethylene diisocyanate is inactive but, if the isocyanate groups are replaced by electrophilic groups such as β-chloroethylamine, epoxide, ethyleneimino and mesyloxy, active compounds are in every case obtained.

The similarity of the end effects produced by ionizing radiations which are generally thought to act *via* free radicals formed *in situ* and the alkylating agents has led to suggestions that the latter can also give rise to free radicals during the course of hydrolysis. Butler (1950) has pro-

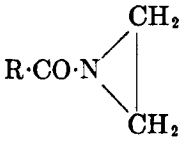
posed that the carbonium ion of a nitrogen mustard may exist as a resonance hybrid in which a relatively important contributing structure is that of a biradical. Jensen (1950) points out that this suggestion as it stands is impossible on quantum mechanical grounds since resonance cannot occur between structures with different numbers of unpaired electrons. Boyland (1952b) proposed that with a bifunctional nitrogen mustard a free radical is produced from one of the arms after the other has reacted with a cell constituent. It is difficult to see a mechanism whereby such a reaction can occur. Apart, however, from these theoretical objections the production of free radicals during the hydrolysis of these alkylating agents has been ruled out by Bonème and Magat (1951), who showed that these compounds do not initiate polymerization which is a most sensitive test for free radicals. Nor could any evidence for free radicals be found by Alexander and Fox (1952a).

Philips (1950) has proposed that the criterion for activity is the presence of an unstable three membered heterocyclic ring. Epoxides and ethyleneimine derivatives automatically fall into this class while sulfur and aliphatic nitrogen mustards form such a ring as an unstable intermediary during hydrolysis. The discovery of activity in the mesyloxy series, however, argues against this hypothesis since these compounds cannot form such ring structures.

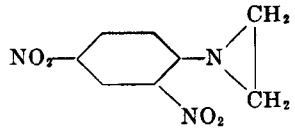
From the foregoing it can be concluded that the presence of electrophilic groupings capable of reacting with anions is a necessary condition for activity though it is not alone sufficient.

B. *Multiplicity of Functional Groups.* In experiments designed to determine the optimum requirements for the marked cytotoxic effects as demonstrated by growth inhibition of tumors Haddow, Kon, and Ross (1948) found that an essential condition for activity in the mustards was the presence of two haloalkyl (i.e., the alkylating centers) groups in the molecule. This conclusion also emerged from the work in other laboratories (cf. Haddow, 1953). The same requirement is operative in the epoxide (Ross, 1950; Hendry *et al.*, 1951b) and mesyloxy (Haddow and Timmis, 1950) series. Certain of the monofunctional compounds can produce some radiomimetic effects, such as breaking chromosomes but almost invariably require fifty to one hundred times the concentration necessary with the corresponding monofunctional compound (Biesele *et al.*, 1950; Loveless, 1951). The toxicity of the alkylating agents precludes their use at such dose levels in the intact animal and no inhibition of tumor growth could be detected by Haddow (1953) in any of the monofunctional compounds when given at concentrations comparable to those of the bifunctional compounds. In general no increase in activity is derived if the molecules contain more than two functional groups.

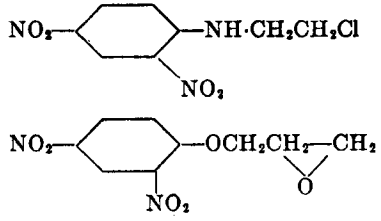
In the ethyleneimine series the superiority of polyfunctional compounds is also very apparent (cf. Hendry *et al.*, 1951c), but here a number of monofunctional compounds have been found growth inhibitory and ethyleneimines carrying an alkyl chain are carcinogenic [e.g.



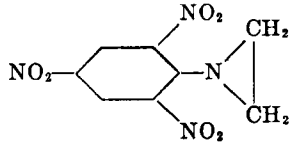
where R is C_5H_{11}, $C_{13}H_{27}$, and $C_{17}H_{35}$ (Hendry *et al.*, 1951c)]. Most effort has been devoted to determine the reasons for the activity of 2,4-dinitrophenyl ethyleneimine



which is comparable to that of many bifunctional compounds. Both Hendry *et al.* (1951c) and Alexander *et al.* (1952) have suggested that the activity may be due to powerful interaction of the dinitrophenyl residue by secondary forces which can take the place of one of the alkylating centers. The fact that monofunctional derivatives carrying such groups can produce effects comparable to crosslinking in fibers provides some support for this view (see p. 20). However, the absence of activity in



argues against this hypothesis as does also the inactivity of



since the trinitrophenyl group is capable of greater interaction by van der Waals forces than the dinitrophenyl group (Hendry *et al.*, 1951c; Haddow, Everett, and Ross, 1951).

A further exception to this rule was found by Stevens and Mylroie (1952), who showed that monofunctional sulfur mustard derivatives were possessed of the same mutagenic activity as the bifunctional parent substance. On the other hand Haddow (private communication) failed to find any growth inhibiting properties with the one-armed sulfur mustard, $C_2H_5SC_2H_4Cl$. The reasons for the activity of the few monofunctional ethyleneimines appears to be a subject worthy of detailed investigation.

C. *Steric Factors.* The relative positions of the alkylating centers in the molecule have been systematically varied. In the series of bis-epoxides $CH_2(O)CH{-}(CH_2)_n{\cdot}CH(O)CH_2$ (Everett and Kon, 1950) the activity falls as n is changed from 0 to 6 and is absent when $n = 16$. Similarly Kon and Roberts (1950) showed that in the modified nitrogen mustard

$$\begin{array}{l} R{\cdot}N{\cdot}(CH_2)_n{\cdot}N{\cdot}R \\ \quad\;\; | \qquad\quad | \\ (CH_2)_2 \quad (CH_2)_2 \\ \quad\;\; | \qquad\quad | \\ \quad Cl \qquad\; Cl \end{array}$$

a decrease in activity occurs when n becomes greater than 3. In the mesyloxy series (Haddow and Timmis, 1950, 1953), $X{\cdot}(CH_2)_n{\cdot}X$ (where X is $CH_3SO_2{\cdot}O{-}$), activity is at a maximum when $n = 4$ or 5, decreases for $n = 6$, 7 or 8 and is only marginal for $n = 2$ or 3, or 9 or 10.

A further condition for activity has been revealed in this series. Haddow and Timmis (1950) found no activity in compounds where the two mesyloxy groups are so placed that they cannot form a ring compound. Thus the introduction of a triple bond that confers complete rigidity on the molecule $X{\cdot}CH_2C{\equiv}C{\cdot}CH_2$ renders the compound inactive. Similarly the *cis* compound $X{\cdot}CH_2CH{:}CH{\cdot}CH_2{\cdot}X$ is active whereas the *trans* compound which cannot form a ring $X{\cdot}CH_2CH{:}CH{\cdot}CH_2{\cdot}X$ is inactive. This led Timmis (1951) to suggest that the biological action depends upon the twofold alkylation of a primary amino group with the formation of a ring

$$-NH_2 + X{\cdot}R{\cdot}X \rightarrow -N(R)-$$

This hypothesis would also explain the inactivity of the monofunctional compounds since these would only dialkylate if present at very high

concentration. The variation in activity in the series $X{\cdot}(CH_2)_nX$ is in agreement with this view since the ring structure will be most stable when $n = 4$ or 5. The differences in the rate of hydrolysis of these compounds (see Table I) can also be explained on the basis of steric interaction between the groups. However, the rate of hydrolysis of the higher members is the same as that of the most active (i.e. $(CH_2)_4$) compound while their biological activity progressively diminishes. The absence of a parallelism between these two factors suggests that other criteria besides the presence of two alkylating centers must be fullfilled for biological activity.

TABLE I

Rates of Hydrolysis of $CH_3SO_2O{\cdot}(CH_2)_nO{\cdot}SO_2{\cdot}CH_3$ in 50% Acetone-Water at 60°C. (Hudson, Marshall, and Timmis, 1953)

n	Rate $\times 10^3$ hrs.$^{-1}$
2	2.32
3	19.7
4	65.8
5	59.6
6	60.7
7	66.8
8	61.4
9	61.3
10	59.4

The decrease in activity on increasing the separation of the two halogenoalkyl and epoxide groups (see p. 10) within the same molecule is also in accord with the ring closure hypothesis, which cannot, however, be put to the same decisive test here as with the mesyloxy compounds since the site of reaction is always at the end of a flexible two-carbon chain. It is relevant to mention here that there is ample evidence (cf. Ross, 1953) for the formation of a cyclic tertiary amine when sulfur or nitrogen mustards react with primary amines. The spatial configuration required to form such a ring and to alkylate adjacent phosphate groups in nucleic acid (see p. 31) is similar, and the relationship between biological activity and disposition of the two alkylating groups would also lend support to this hypothesis.

D. *Summary.* Pronounced cytotoxic activity in the animal and ability to produce tumors is found in compounds containing two or more alkylating groups capable of reacting with nucleophilic groups. A probable condition for activity is that the two reacting groups are so placed that they can form a stable ring. The structure of the remaining part of the molecule appears to be of qualitative importance only. Thus active compounds

have been obtained which are neutral, acidic, or basic and which contain almost every type of aromatic or heterocyclic ring structure. Only if the substituents introduce steric restriction or influence the chemical reactivity of the alkylating groups (cf. Ross, 1953) can any pronounced difference in biological behavior be detected. This almost limitless variety of active structures eliminates the possibility that specific adsorption processes play a part in their mechanism of action which can probably be ascribed solely to a chemical reaction with vital centers, possibly situated in the cell nucleus. This does not, of course, imply that the therapeutic value of these substances cannot be varied by attaching the alkylating centers to different groups which will influence important ancillary factors such as membrane permeability.

2. Competition Factors of Macromolecules

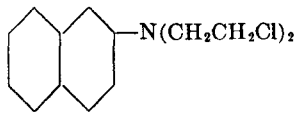
The cytotoxic alkylating agents have been shown *in vitro* experiments to react with very many substances of biological interest (e.g., Fruton *et al.*, 1946), and the relative importance of the different reactions in biological systems can be judged from the consideration of the so-called competition factor of the reacting substance. All the alkylating agents hydrolyze in water and the rate of this reaction has been used as a general criterion for their reactivity (cf. Ross, 1953); if another substance (A) capable of reaction is also present then the ratio

$$\frac{\text{Rate of reaction of A}}{\text{Rate of hydrolysis}} = \frac{\text{Amount of A reacted}}{\text{Amount of hydrolysis} \times \text{Concentration of A}} = F_A$$

is referred to as the competition factor (Ogston, 1948). In a complex system the relative amounts of the different constituents alkylated depends therefore on both these concentrations and F_A, competition factor. In agreement with the general ideas developed on p. 6 it is found that groups capable of donating an electron (i.e., nucleophilic) have a high competition factor. It has not, however, proved possible to explain on theoretical grounds the relative magnitude of the competition factors of different substances. For sulfur mustard Ogston (1948) found thiol compounds and particularly thiophosphonates to have competition factors varying from 10^3 to 10^5 due undoubtedly to the lone pair of electrons on the S atoms which are highly nucleophilic; anions of organic acids have competition factors varying from 10 to 100. Ogston records low competition factors for amines (i.e., 5 to 20), but this is undoubtedly due to the fact that working at pH 7 to 8 the amines were in the cationic form when they are not nucleophilic. Since the lone pair of electrons on a nitrogen atom are strongly nucleophilic, it is to be expected that un-ionized bases will have very high competition factors.

Competition factors have been determined by Ross (1949) for the reaction between an aromatic nitrogen mustard and a number of anions and thiol compounds, which were placed in approximately the same order as for mustard gas. A competition factor as defined above cannot be determined for epoxides since the kinetics of their reaction are different from those of the mustards. Ross (1950), however, has determined the relative activity of a number of anions and found that those are placed in an order not far different from those of the mustards although the differences in magnitude are much smaller.

With mustard gas Ogston (1948) found that a phosphate group in a nucleic acid had a competition factor about four times greater than that of a simple inorganic phosphate. This difference is unlikely to be the result of combination with an organic residue since the competition factor of glycerol phosphate is low. Alexander and Fox (1952a) found with an aliphatic nitrogen mustard that the competition factor of a carboxyl group in a very high molecular weight polymethacrylic acid was substantially greater than that of simple organic acids; for example, in a solution 0.003 *N* with respect to carboxyl groups 80% of the mustard had reacted by esterification and less than 20% had hydrolyzed. Using the corresponding monomer these figures were reversed. These experiments were repeated with the aromatic nitrogen mustard



(Alexander and Ross, 1951), and it was found that the polymethacrylic acid had a competition factor of between 1000 and 3000, depending on the conditions of the experiment, whereas simple organic anions have values ranging between 50 and 150. There are preliminary indications that the competition factor increases with increasing molecular weight of the polymer, but no detailed figures have yet been obtained. These results would suggest that the abnormally high competition factor of nucleic acid is also due to the presence of the anions in a macromolecule. This work supports the view that the biological mechanism depends upon reaction with ionized macromolecules and would explain why the alkylating agents are active in *in vivo* when experiments with small molecules indicate that they would be almost wholly destroyed by hydrolysis at the low concentrations used.

These considerations do not apply to proteins which combine either equally or less readily than their constituent amino acids with mustard gas; thus the competition factor of an amino acid residue basis of serum

albumin as for mustard gas, is 40 as opposed to 1100 for phosphoric acid (Ogston, 1948). The much greater reactivity of nucleic acid compared with proteins, evident from their competition factors, was demonstrated by Herriott's (1948) studies of the inactivation of enzymes and viruses. He finds that the rate of inactivation of viruses and cells by mustard gas to be of the same order but faster than those of enzymes. Of the viruses examined those containing deoxyribonucleic acid were inactivated faster than those containing ribonucleic acid. Preparations of the pneumococcus transforming principle which were largely deoxyribonucleic acid were the most easily deactivated of all the systems examined. Banks *et al.* (1946) and Young and Campbell (1947) both found that significantly more mustard gas had combined with nucleoproteins than with serum proteins, prolamines, such as zein and gliadin, and keratins. Boursnell *et al.* (1946) noted that the tissues most severely damaged (e.g., bone marrow) contained much less bound radiosulfur after treatment with radioactive mustard gas than many of those which had suffered least damage, and were led to conclude that a general fixation to protein was not the primary cause of the systemic effects of mustard gas. In disagreement with these results Carpenter *et al.* (1948) found that mustard gas combined to approximately the same extent with the protein as with the ribonucleic acid of tobacco mosaic virus. However, this virus which contains 94% of protein is, according to Herriott (1948) the virus least sensitive to mustard gas.

In a direct comparison of mustard gas with aliphatic nitrogen mustards Herriott (1948) found that the coefficient—log (decrease in biological activity of virus) per 10^{-3} M agent—is the same for mustard gas as for $CH_3N(CH_2CH_2Cl)_2$ and $C_2H_5N(CH_2CH_2Cl)_2$ but that $N(CH_2CH_2Cl)_3$ is two to three times as effective. Similar data concerning the relative reactivity of proteins and nucleic acid are not available for any of the other cytotoxic alkylating agents but considerations of their reactivity with macromolecules (*vide infra*) do not indicate any qualitative differences.

*3. The Crosslinking Hypothesis**

Having established that one of the outstanding requirements for biological activity of the nitrogen mustards is the presence of two halogeno alkyl groups per molecule, workers at the Chester Beatty Research Institute (Goldacre, Loveless, and Ross, 1949; Haddow, 1949) put forward the suggestion that the biological activity depends on the reaction of the same molecule with two centers of a biological macromolecule. These points of attachment could be either on the same chain which may,

* Frequent reference will be made in this and succeeding sections to reactions with the nitrogen mustard $CH_3N(CH_2CH_2Cl)_2$, and this will be referred to throughout as HN2. The compound $S(C_2H_4Cl)_2$ will be referred to as mustard gas.

under certain conditions, lead to changes in shape of the molecule (see p. 17) or they may be on two different chains in which case crosslinking takes place. The cytological abnormalities such as chromosome fragmentation, bridge formation at anaphase and chromosome "stickiness" which are such prominent features of the cytotoxic alkylating agents could be explained by crosslinking of chromosome threads prior to mitosis. The new covalent link thus formed if stronger than the fibers themselves—these may consist of strings of globular molecules held together only by hydrogen bonds and secondary forces as in fibrin (Porter and Hawn, 1949) or F-Actin (Perry and Reed, 1947)—leads to the breaking and the other macroscopic abnormalities of the chromosomes which are observed. Consideration of these principles led to the discovery by the group at the Chester Beatty Institute (see p. 4) of cytotoxic activity in the bis-epoxides and polyethylenimines which were examined because of their

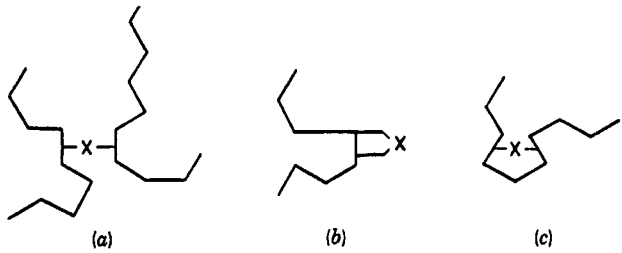


FIG. 1. Different types of reactions of a bifunctional compound with macromolecules: (a) intermolecular crosslinking; (b) intramolecular reaction on neighboring groups, (c) internal crosslinking within a coiled molecule.

known ability to crosslink wool and cellulosic textile fibers respectively (see p. 18). With the techniques at present available, it is not possible to test this hypothesis directly by investigating chemically the reactions with chromosomes and experiments are confined to studies in model systems which cannot provide conclusive evidence.

A. *Inter- and Intramolecular Reaction.* There are essentially three ways in which bifunctional reagents can react with a macromolecule (see Fig. 1a, b): (1) intermolecularly to form crosslinks between different chains; (2) intramolecularly to react with neighboring groups along the same chain; (3) intramolecularly to react with groups in different parts of the same molecule, thus bringing about internal crosslinking. This last reaction can occur only if the molecule is linear and flexible as is the case for many synthetic polymers dissolved in a good solvent. The various segments of such a molecule can move relative to one another and the molecule will vary continuously in shape and Plate 1 (taken from Kuhn and Kuhn, 1948) shows a typical configuration of a long chain aliphatic

hydrocarbon. Internal molecular crosslinking is not possible with rigid molecules such as globular proteins or inflexible rods such as tobacco mosaic virus.

In a given system a change-over from an intra- to intermolecular reaction will occur as the concentration of the macromolecules in solution is increased. This effect was demonstrated by Alexander *et al.* (1952) with serum albumin when it was found, using light scattering, that HN2 and a bis-epoxide at a concentration of 10^{-2} M will bring about an increase in the average molecular weight from 75,000 to 210,000 and

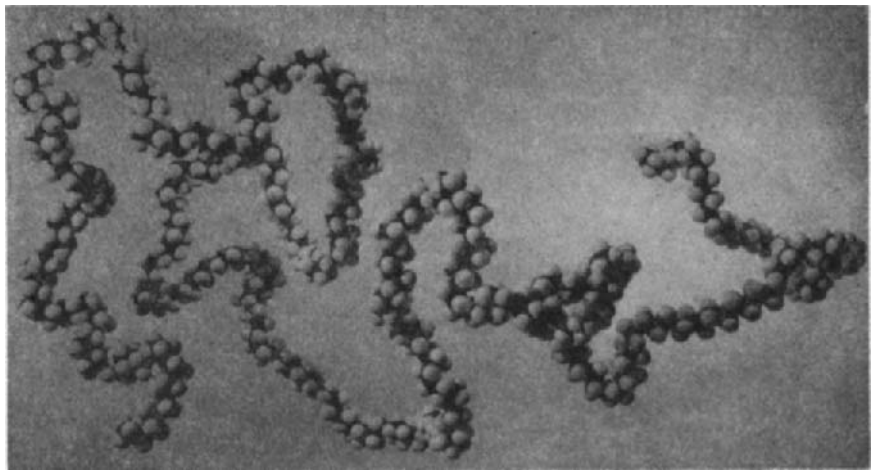
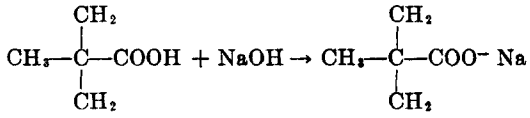


PLATE I. Shape of a hydrocarbon ($C_{500}H_{1002}$) molecule in benzene solution in which it is coiled at random. (Courtesy Prof. W. Kuhn.)

170,000, respectively, if the protein is at a concentration of 2% but that no increase occurs if the reaction takes place in 0.5% solution. At the lower concentration, however, the protein still reacts as shown by the changed slope of the curve τ/C versus C (where C is the concentration of the protein and τ the intensity of the scattered light), but the reaction must have proceeded exclusively intramolecularly. A similar observation was made by Butler (1951), who found after treatment of a 0.2% solution of serum albumin with HN2 one peak only in the ultracentrifuge diagram, but after treatment of 2% and 20% solutions two and three peaks, respectively, which indicate the presence of higher molecular weight species.

The change-over from inter- to intramolecular crosslinking is clearly illustrated in experiments (Alexander and Fox, 1952a) with polymethacrylic acid (PMA). This material is coiled as shown in Plate I, but on neutralization becomes expanded into a linear structure due to the mutual repulsion of the ionized carboxyl groups. This change is accompanied by

a several hundred-fold increase in viscosity. A coiled structure can also be obtained from the ionized form by adding electrolyte which reduces the repulsion between the charged groups. If PMA is treated with HN2,



its viscosity in the ionized form is very greatly depressed (see Fig. 2). This decrease was shown to be due to internal crosslinking which prevents the coiled form from expanding on neutralization to its full length by the fact that the increase in viscosity on going from acid to salt form is much

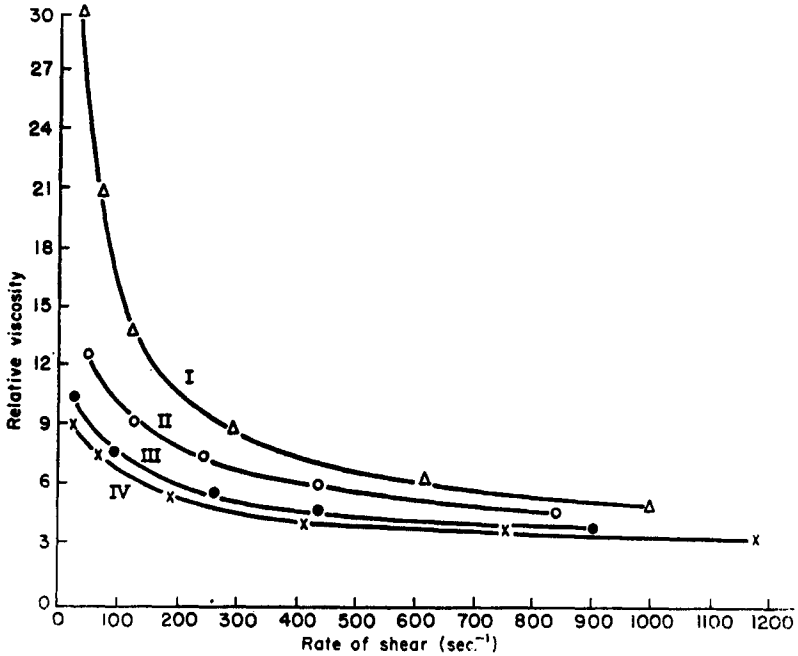


FIG. 2. Change in viscosity of high molecular weight polymethacrylic acid after treatment with different quantities of HN2: I viscosity of 0.04% polymer at pH 7; II after reaction with a solution of 0.0085% of HN2; III after reaction with a solution of 0.0213% of HN2; IV after reaction with a solution of 0.042% of HN2.

less in HN2 treated than in untreated PMA. The whole of this effect can be reversed by saponifying the ester crosslinks with alkali when the PMA regains its original viscosity. If the reaction with HN2 is carried out in progressively more concentrated solutions the fall in viscosity becomes less until the whole effect is reversed and an increase in viscosity is

observed. Finally, with 2% solutions of PMA HN2 brings about the formation of a gel (Alexander, unpublished). This is another example of the change-over from an intra- to an intermolecular reaction as the concentration of the marcomolecule is increased.

With a rigid linear molecule in which the reactive groups along the chain are at a considerable distance apart, intramolecular reaction is not possible since there are no two centers on the same molecule stearically accessible to the bifunctional reagent. Under these conditions intermolecular reaction (i.e., crosslinking) is favored. A reaction of this type is the crosslinking with mustard gas of high molecular weight sodium alginate, a comparatively rigid molecule having repeating carboxyl groups at 10 A. apart, which Deuel and Neukom (1949) found to form gels at a concentration of less than 0.1%. At this concentration the reaction with PMA is exclusively intramolecular.

Although the effect of concentration on the reaction of deoxyribonucleic acid with mustard gas has not been determined systematically, a change-over from intra- to intermolecular reaction appears to occur as the concentration is increased. Elmore *et al.* (1948) noted a considerable increase in molecular weight with a 35% solution of nucleic acid, whereas with solutions containing less than 1% the reaction with mustard gas and HN2 is now thought to be intramolecular (see p. 26).

Under biological conditions all the different reactions considered here can take place, and it is not inconceivable that they may occur simultaneously. In this case some of the biological effects may possibly be produced by an inter- and others by an intramolecular reaction. With different bifunctional agents steric factors may favor one kind of reaction more in one case than in another and this could bring about the differences in the biological effects produced by the different compounds.

B. *Comparison of Crosslinking Ability and Biological Activity.* The ability of the mustards to crosslink proteins has already been described. Bis-epoxides were found to crosslink alginic acid and pectin by Deuel (1947), and wool by Capp and Speakman (1949; Fearnley and Speakman 1950). Many polyethyleneimines had been prepared during the war in the I.G. Farben Laboratories in Germany, and general methods of preparation have been worked out there (Bestian, 1950). Interest in these compounds arose since they were found to increase the wet tensile strength of regenerated cellulose fibers. It is known that such a change can be brought about either by crosslinking the cellulose molecules or by depositing a polymer within the fiber and the I.G. scientists (see report by Alexander and Whewell, 1946) were in disagreement as to the reaction which occurred on treatment with the polyethyleneimines. With wool it was established that both reactions occurred; with the tris-ethyleneimines

triazine polymer formation on the surface was seen on microscopic examination of the fibers, and the formation of new crosslinks was also established (see below).

To determine whether the ability to crosslink a protein runs parallel with the biological activity of these compounds Alexander *et al.* (1952c) examined their reaction with wool fibers. From the results summarized in Table II no correlation can be seen between crosslinking and cytotoxic

TABLE II

Crosslinking of Wool Fibers with Biologically Active and Inactive Alkylating Agents (Alexander *et al.*, 1952)

Tumor Growth Inhibitors	Extent of Crosslinking
$CH_3N(CH_2CH_2Cl)$ (HN2)	−
$S{\cdot}(CH_2CH_2Cl)_2$	−
p-$CH_3O{\cdot}C_6H_4N(CH_2CH_2Cl)_2$	+
Trisethyleneiminotriazine	+++
$(CH_2)_2N{\cdot}CON(CH_2)_6NCON(CH_2)_2$	++
bis—2,3-epoxypropyl ether	+++
$CH_3SO_2{\cdot}O{\cdot}(CH_2)_3O{\cdot}SO_2CH_3$	++
$CH_3SO_2O{\cdot}(CH_2)_4{\cdot}OSO_2CH_3$	++
Not active as tumor growth inhibitors	
$CH_3SO_2O{\cdot}CH_2C{\equiv}CCH_2{\cdot}OSO_2CH_3$	+++
$CH_3SO_2O{\cdot}CH_2{\cdot}\overset{\overset{CH_3}{\vert}}{\underset{\underset{NO_2}{\vert}}{C}}{\cdot}CH_2{\cdot}OSO_2CH_3$	+++
Epichlorhydrin	+++
1,5-Difluoro-2,4-dinitrobenzene	+++
1-Fluro-2,4-dinitrobenzene	+

activity. In the mesyloxy series those compounds which cannot inhibit tumor growth and which cannot form cyclic compounds (see p. 10) are amongst the best crosslinking agents examined. It is interesting that the aliphatic mustards fail to crosslink wool although they will crosslink soluble protein.

The experiments with wool fibers do not, of course, constitute proof against the crosslinking hypothesis, although in the author's opinion they detract from the support which had been deduced from the fact that the cytotoxic alkylating agents were all capable of crosslinking macromolecules in one system or another. A further argument adduced by Hendry *et al.* (1951b) against this hypothesis is that activity is found both in substances in which the two reactive centers are close to one another and when they are 15 to 20 A. apart. They conclude that if the biological

effects were due to the formation of bridge a wide range of distances between the points of attachment would have to be admitted. This objection does not, however, appear sound since, in the compounds considered by Hendry *et al.* in this connection, the chain separating the reactive groups is flexible and would permit the molecule to take up a large number of different configurations.

It is interesting that compounds with only one reactive group but containing a 2,4-dinitrobenzene group (see Table II) or long alkyl chain, such as heptyl (Patterson *et al.*, 1940) bring about effects in wool fibers comparable to those of covalent crosslinking. This is probably brought about by the strong secondary forces which would bind these groups to one another or to existing groupings in the fibers. The 2,4-dinitrobenzene and long chain alkyl derivatives of ethylene imine are the most potent monofunctional substances which are cytotoxic in the intact animal and it is tempting to draw an analogy between the two systems; it is doubtful if this is permissible in view of the results with compounds described on p. 9.

C. *Crosslinking and Aging.* Recently Haddow (1953) has proposed an extension of the crosslinking hypothesis to the occurrence of spontaneous tumors. Observing that the deterioration of plastics, rubbers and proteins on aging is often due to the formation of crosslinks between chains he speculates whether the increasing incidence of cancer with old age may result from a similar deterioration of tissue components. In view of the large number of potential crosslinking agents (e.g., aldehydes) which are normally present or formed in the body, it is not unlikely that during metabolic processes the formation of crosslinked structures occurs as a side reaction (Bjorksten, 1951), and there may be a special biochemical mechanism whereby these are eliminated. As the animal gets older, there is evidence that the rate of breakdown of proteins becomes less (cf. Neuberger *et al.*, 1951), and it is possible that crosslinked structures may then accumulate. Alternatively with decreased rate of metabolic turnover there may be time for the crosslinking process to proceed to such an extent that the product can no longer be broken down in the body. In this connection attention may be drawn to the production of cancer by the introduction of inert films (see p. 65) since their effect might be comparable to the accumulation of a highly insoluble metabolic product.

4. *The* in situ *Polymerization Hypothesis*

Hendry *et al.* (1950, 1951a,b,c) suggest that the cytotoxic alkylating agents polymerize *in situ* to give a macromolecule containing reactive groups. The necessity for two functional groups, according to this hypothesis, arises from the fact that one brings about the joining up of

the molecules into a polymer and the second group provides the reactive centers in the polymer. The types of structure postulated for bis-epoxides, bis-ethyleneimines and nitrogen mustards are shown in Figs. 3, 4, and 5, respectively. These authors stress that the separation of reactive centers in polymers derived from the three classes of compounds is the same

(III)

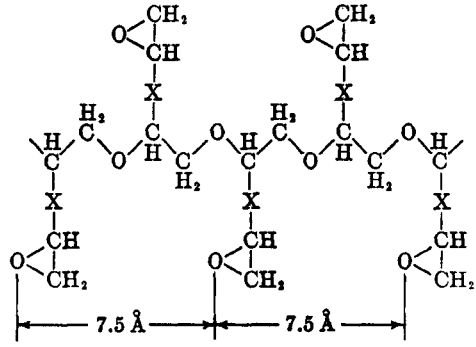


FIG. 3. Structure postulated for the reactive polymer formed *in situ* from bis-epoxides (Hendry *et al.*, 1951b).

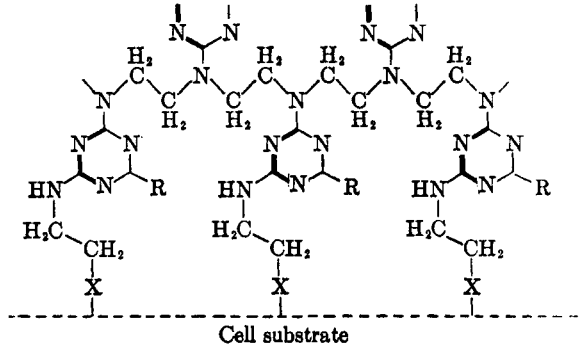


FIG. 4. Structure postulated for the reactive polymer formed *in situ* from bis-ethyleneimine (Hendry *et al.*, 1951c).

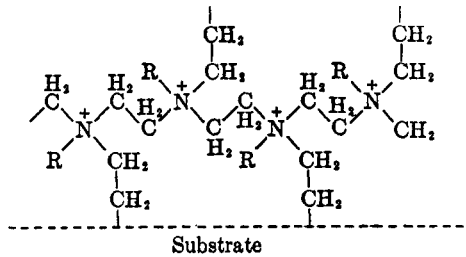
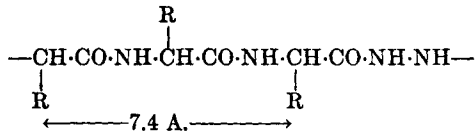


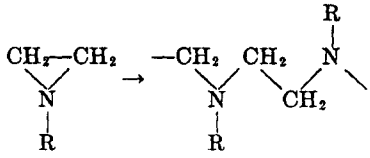
FIG. 5. Structure postulated for the reactive polymer formed *in situ* from a nitrogen mustard (Hendry *et al.*, 1951c).

and that this distance is independent of the nature of the group linking the two reactive centers. In this they see the explanation for the great similarity in biological activity of compounds possessing such widely divergent structures. Further importance is attached to the fact that the distance at which the reactive groups repeat is approximately 7.4 A., which is close to the repeat distance of the side chains of a fully extended protein chain. Hendry *et al.* indicate a number of ways by which these

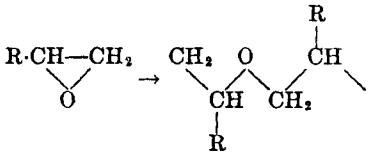


hypothetical structures act in the cell to bring about the observed chromosome abnormalities and cytotoxicity. One suggestion is that they polymerize and that the side chains then bring about crosslinking with multipoint attachment of two protein or nucleoprotein chains of chromosomal origin. Another suggestion is that one arm of the monomeric form reacts with a cell compound followed by self condensation via the second reactive group.

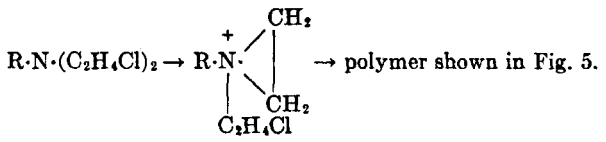
The polymerization hypothesis finds a measure of support from the fact that ethylene imines can polymerize under mild conditions according to this reaction:



The corresponding reaction with epoxides has also been recorded though only in alkaline media. For the polymerization of the mustards via their



ethyleneimonium form there is considerably less evidence. Hanby and Rydon (1947) found that HN2 dimerized but failed to detect higher polymers. Moreover, with the aromatic mustards Ross (1953) could not detect the intermediate ethyleneimonium ion which must therefore be very unstable and polymerization according to the following mechanism



will therefore be confined to the aliphatic mustards.

Moreover, aromatic mustards of the constitution COOH $(CH_2)_n \cdot C_6H_4 \cdot N\ (C_2H_4Cl)_2$ which include some of the most active compounds known (Everett, Roberts, and Ross, 1953) readily polymerize at pH 7 according to the following reaction:

$$2COOH(CH_2)_nC_6H_4(C_2H_4Cl)_2 \rightarrow$$

$$COOH(CH_2)_nC_6H_4 \cdot \underset{\displaystyle CH_2CH_2Cl}{\overset{|}{N}} \cdot CH_2CH_2OOC(CH_2)_nC_6H_4 \cdot N(C_2H_4Cl)_2$$

These polymers do not of course fulfil the criteria of 7.4 A. repeat distances required by the theory under discussion, while the ease with which they are obtained would appear to preclude the formation of the polymer shown in Fig. 5.

Even with the ethyleneimines which polymerize so readily, there is no evidence that polymers of the constitution shown in Fig. 4 can be obtained. When polyethyleneimines are polymerized a highly insoluble network is invariably obtained, which results from crosslinking by spare functional groups on one chain with those of another chain, and the resulting structure bears no resemblance to that postulated. Furthermore, general considerations suggest that in a biological system where there are a very large number of compounds capable of reacting with the cytotoxic alkylating agents, these would in the high dilution at which they are active react with cell components in preference to self-condensation. In model experiments which simulate these conditions no polymer formation could be detected with the mustards, the epoxides, or even the ethylene imines (Alexander, unpublished).

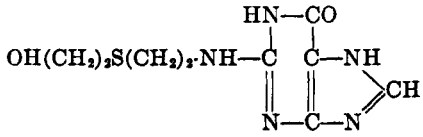
For the mesyloxy compounds it is chemically impossible to polymerize to structures resembling those postulated by Hendry *et al.* for the other types of cytotoxic alkylating agents. The only conceivable polymer which could be formed from $CH_3SO_2 \cdot O \cdot (CH_2)_n \cdot OSO_2CH_3$ would be a hydrocarbon, $—(CH_2)_n \cdot (CH_2)_n—$, which does not fulfill the requirements of the theory. However, even such a polymerization is most unlikely, and no evidence for it could be found. Unless the mesyloxy compounds act by a different mechanism from that of the other alkylating agents, which is improbable in view of the general chemical similarities, the polymerization hypothesis will be difficult to sustain.

5. Reaction with Nucleic Acids

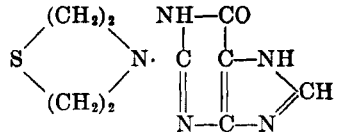
A. *Site of Reaction.* Reference has already been made to the readiness with which nucleic acids react with mustard gas and HN2. From general considerations of the reactivity of these compounds reaction at pH 7 is possible in nucleic acids with the primary and secondary phosphate groups which are fully dissociated and the amino groups of adenine, cytidine, and guanine; the last group, in view of its low basicity, is not very electrophilic and is thus unlikely to have high competition factors. Neither the sugar hydroxyls nor those from uracil and thymine are likely to react since they are completely associated (i.e., in the non-reactive form) at pH 7. In agreement with these predictions Elmore *et al.* (1948) found that the sodium salts of both deoxyribo (DNA) and ribonucleic acids (RNA) reacted readily with mustard gas and that the major reaction was esterification of the phosphate groups. They also concluded that some of the amino groups had reacted, though an estimate was not possible; contrary to expectation they found indication that some of the purine-pyrimidine hydroxyl groups had also reacted. These findings were based on changes in the electrometric titration of the nucleic acids after reaction and are not conclusive since certain parts of the titration curves cannot be reliably interpreted. The evidence for the esterification of the phosphate groups is unambiguous and was confirmed by analyses for sodium. Total sulfur analyses indicate that with DNA the majority of the mustard gas had reacted with two groups in the nucleic acid whereas with RNA reaction with one arm only predominates leaving the residue $—CH_2CH_2—S·CH_2CH_2·OH$. In agreement with these analyses it was found that extensive crosslinking leading to a product of increased molecular weight was obtained with DNA. With both nucleic acids it was found that as the reaction with mustard gas proceeded part of the nucleic acid was precipitated from the solution. All the analyses discussed above refer to the soluble fraction, and no detailed study of the other fraction was made, the insolubility of which remains unexplained.

Alexander (1952) provided evidence that all the cytotoxic alkylating agents esterify the phosphate groups of DNA (see p. 29), although the rate of reaction was much slower with the epoxides and mesyloxy compounds than for the mustards and ethyleneimines. Also more of the epoxides and mesyloxy compounds (i.e. in higher concentrations) have to be used than of the mustards and ethyleneimines to achieve the same extent of esterification, probably because the proportion of the former, which hydrolyze instead of reacting with the DNA, is larger (Alexander, unpublished).

Young and Campbell (1947) treated a number of purines and pyrimidines with mustard gas and quantitatively converted guanine into two compounds containing 11.7 and 11.8% of sulfur which they concluded to be



and



Reaction with adenine was very slow and, as was to be expected, no combination took place with uracil.

HN2 like sulfur mustard reacts with the purines and pyrimidines of nucleic acids. This was first demonstrated spectrographically (Chanutin and Gjessing, 1946), and more recently a group in the Southern Research Institute (1952) using a new chromatographic separation technique showed that at least two different reaction products are formed with each purine. Reaction also occurs with the purines and pyrimidines of intact nucleic acid (Butler and Press, 1952); after extensive reaction with HN2 followed by acid hydrolysis the number of total amino groups (estimated by Van Slyke nitrogen) fell by 10% though the proportion of purines precipitated by silver salts decreased more. Since the extent of modification of the purines required to prevent precipitation with silver salts is not known, no conclusion concerning the nature of the reaction can be made. The limited data available, however, suggest that the reaction of HN2 with DNA is similar to that with mustard gas and consists of esterification of phosphate groups and some unknown reaction with amino, and possibly also hydroxyl groups of the purines and pyrimidines.

B. *Decrease in the Viscosity of Deoxyribonucleic Acid.* Aqueous solutions of the sodium salt of DNA, which has been isolated under conditions which minimize degradation, behave as non-Newtonian liquids; that is, their viscosity increases with decreasing rate of shear (see Fig. 6). Gjessing and Chanutin (1946) observed that after treatment with HN2 the viscosity of DNA was markedly reduced; J. A. V. Butler and his colleagues (Butler, Gilbert, and Smith, 1950; Butler and Smith, 1950; Conway, Gilbert, and Butler, 1950) extended these investigations and showed that sulfur mustard acted similarly and that the effect was most pronounced at low rates of shear (i.e., where the viscosity was most anomalous). In general the viscosity of a solution of a macromolecule is a direct function of

its molecular weight and consequently both groups of investigators were originally led to the conclusion that the mustards degrade DNA, and this appeared to be supported by independent molecular weight determinations from sedimentation and diffusion constants. This deduction seemed all the more attractive since x-rays had been shown in 1948 to degrade DNA, and a chemical analogy in the action of ionizing radiations and the cytotoxic alkylating agents appeared to be established, the biradical

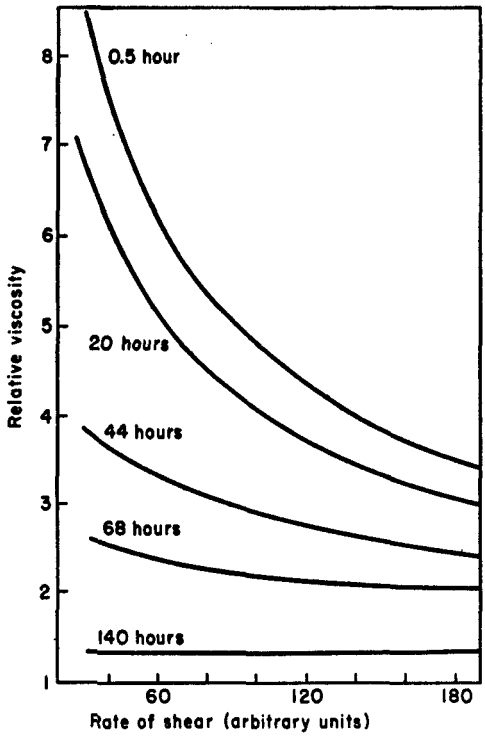


FIG. 6. Decrease in viscosity of deoxyribose nucleic acid (0.1% soln.) after different times following on treatment with HN2 (0.046% soln.) (Butler and Smith, 1950).

theory being proposed (see p. 48) to explain the degradative action of the mustards (Butler, 1950). More recent work has shown that the deduction that the alkylating agents depolymerize DNA is invalid and that the observed changes in the physical properties have a different explanation. A discussion of the factors which are responsible for non-Newtonian viscosity is necessary for an understanding of this important reaction.

The increase of viscosity with decreasing rate of shear of dilute DNA solution is of the same order of magnitude as that found in certain rela-

tively concentrated solutions of colloids where it results from the formation of network-like aggregates which are broken down in streaming. This behavior is accurately described as structural viscosity and has been treated quantitatively in a number of cases (Kuhn, 1932). Changes of viscosity with shear of a very much smaller order of magnitude have been found in solutions of synthetic high polymers, and many attempts have been made to study these cases theoretically, but only the more recent treatment of Kuhn and Kuhn (1945) appears to be satisfactory and in accord with experiment. These authors showed that for asymmetric particles which are randomly coiled and easily deformed the viscosity is independent of shear, whereas for particles which resist deformation shear-dependence is to be expected. However, even with rigid rodlike particles, the viscosity would not decrease as a result of orientation to less than half on going from zero to high rates of shear. Variations of this order have been observed for polymers the molecular weight of which does not exceed a few hundred thousand and show clearly that orientation of the molecules is not sufficient to explain the magnitude of the anomaly found with molecules such as DNA for which a different mechanism must be operative.

A number of naturally occurring macromolecules such as tobacco mosaic virus, hyaluronic acid, fibrinogen and myosin, and the cytoplasmic protein of algae have similar viscosity characteristics. All these substances consist of highly asymmetric molecules which are many thousand angstroms A. long, and this means that the molecules will interact even in solutions, containing less than 0.01% of polymer. Pfeifer (1936) was the first to ascribe the pronounced non-Newtonian viscosity to localized interaction, which gives rise as shown in Fig. 7, to a network of interlocking points. These solutions have been referred to as "gel solution," but this term should not be used (Meyer, 1951) here, since it is correctly applied to solutions which have a definite yield point (i.e., when there is a minimum force necessary below flow occurs), which is not the case for the polymers under consideration.

Alexander and Hitch (1952) studied the viscosity of polymethacrylic acid (PMA) and found that samples of very high molecular weight (*ca.* 2×10^6) had viscosities in extremely dilute solutions, showing the same characteristics as those of DNA (see Fig. 2, p. 17). The shape of a PMA molecule can be altered in steps from an almost fully extended rod to an expanded random coil and finally to a tightly collapsed particle (see p. 31) and this material is therefore ideal for studying the factors influencing non-Newtonian viscosity. It was found that the viscosity became less shear dependent when interaction between molecules was reduced by decreasing the molecular weight or by coiling up the mole-

cule by reducing its charge (i.e., by acidifying) or by decreasing intramolecular electrical repulsion with salts. The non-Newtonian viscosity was also decreased by adding hydrogen bond breaking reagents; these do not change the shape of the molecule but prevent the formation of intermolecular bonds by secondary valencies.

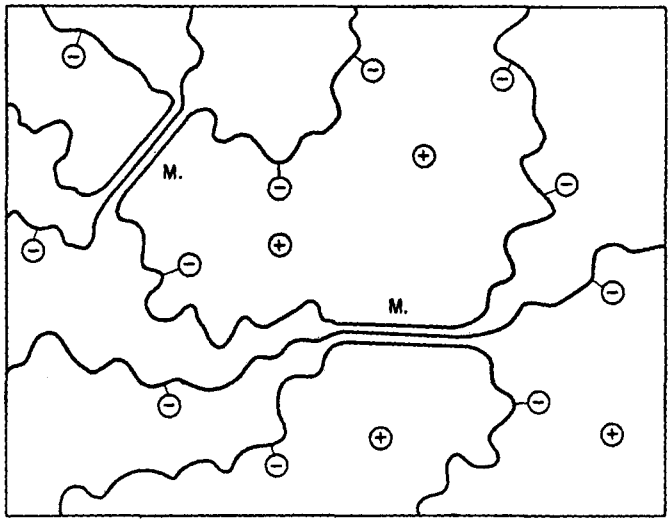


FIG. 7. Diagrammatic representation of interacting macromolecules giving high-viscosity solutions: ⊖ anions fixed on macromolecule; ⊕ gegenions in solution; *M*, points of interaction (Pfeiffer, 1936).

Unfortunately all the hydrodynamic methods for molecular weight determinations are valid only if there is little or no molecular interaction in the solutions used, therefore the same factors which prevent direct deductions from viscosity determinations also make it impossible to determine molecular weights from measurements of sedimentation and diffusion since the absolute values for the constants cannot be found even with the lowest concentrations of DNA at which the conventional instrument can function (cf. Kahler, 1948; Butler and James, 1951).

From the foregoing it is obvious that the only valid conclusion which can be drawn from the physical changes produced by the mustards, such as decrease in viscosity and rate of sedimentation and increase in rate of diffusion, is that the extent of interaction between DNA molecules in solution is reduced. Depolymerization of the macromolecule as for example with x-rays, is only one way by which this can be brought about, but this seems an unlikely mechanism for the mustards since they do not form free radicals (see p. 8), and their primary reaction is one of alkylation. This led Alexander (1950, 1952) to propose that the mustards

change the shape of the DNA molecule. This suggestion was supported by experiments with high molecular weight polymethacrylic acids (PMA), solutions of which in certain of their physical properties closely resemble DNA (Alexander and Hitch, 1952). Experiments which have already been described (p. 17) established that HN2, by producing intramolecular crosslinks, changes the shape of PMA molecules and thereby brings about a change in viscosity similar in every respect to that obtained with DNA (Alexander and Fox, 1952a). Alternatively, if a DNA molecule is flexible and maintained in the highly asymmetric extended form by electrical repulsion, a partial coiling-up could occur by reaction of bifunctional alkylating agents with neighboring phosphate

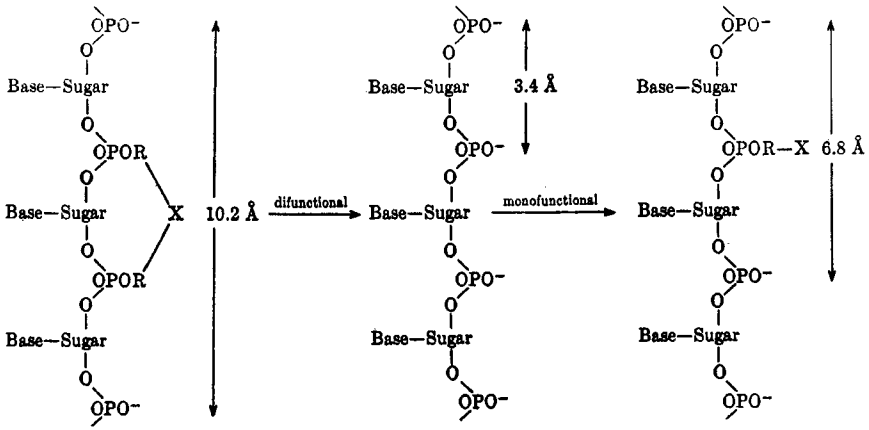


FIG. 8. Postulated reaction of bifunctional alkylating agents with deoxyribose nucleic acid (Alexander, 1951).

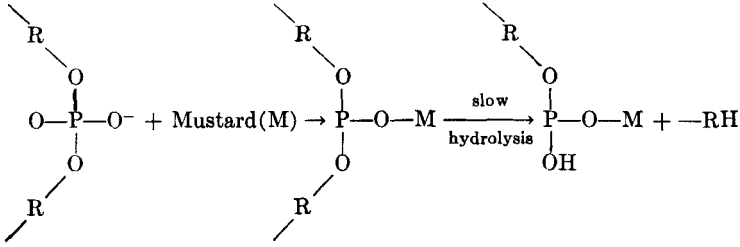
groups (see Fig. 8). This possibility will be considered in more detail on p. 31.

Physical evidence concerning the rigidity of DNA molecules is conflicting. Recent light scattering data (Reichmann, Bunce, and Doty, 1953) indicate that the molecule is flexible and on acidification (i.e., decrease in net charge) coils up; a similar conclusion was also reached by Alexander and Hitch (1952). On the other hand, birefringence studies (Schwander and Cerf, 1951) and measurements of the rotary diffusion coefficient (Benoit, 1950, 1951) do not appear to be compatible with the view that a DNA molecule can coil up. If the molecule is rigid, then the decrease in viscosity may be due to reaction by the mustards with the groups which are responsible for forming the intermolecular bonds which leads to the network structure responsible for non-Newtonian behavior. Greenstein and Jenrette (1941) (see also Conway and Butler, 1952) showed that urea decreased the viscosity of DNA, and from this it can be

deduced that the intermolecular links are formed by hydrogen bonds, the formation of which can be prevented by blocking the amino and hydroxyl groups. This mechanism was favored by Butler, Conway, and James (1951) but seems difficult to sustain in view of the fact that mustards react with no more than 10% of the available amino groups (see p. 25), and this would not be expected to interfere significantly with the formation of intermolecular hydrogen bonds.

Schwander and Signer (1951) proposed that DNA is rigid because of the powerful secondary forces between the nucleotide residues but that the molecule can become flexible without being depolymerized if this interaction is disturbed. Butler, Gilbert, and James (1952) now believe that the action of the mustards is to change the shape of the molecule, though not by one of the mechanisms which involve esterification of the phosphate groups, but by breakdown of intramolecular hydrogen bonds through reaction with amino groups. Attention should, however, be directed to the finding that compounds characterized by ready reaction with amino groups (see p. 7 and Hendry *et al.*, 1951a) are not cytotoxic.

More detailed investigation (Butler and James, 1951) of the physical properties of mustard-treated DNA has definitely established that the initial reaction which produces such pronounced physical changes is not due to depolymerization and is probably brought about in one of the ways discussed above. There are indications (Butler, Gilbert, and James, 1952), however, that the spontaneous degradation by hydrolysis which is known to occur in solution (cf. Taylor *et al.*, 1948) takes place somewhat more readily after reaction with the mustards. A possible explanation for this is suggested by Davis and Ross (1952), who showed that triesters derived from the reaction of a nitrogen mustard with a primary diester phosphoryl group can under certain conditions hydrolyze with fission at the non-mustard linkage. The relative instability of triesters from nucleic acids was demonstrated by Brown and Todd (1952), who showed that main chain fission can arise in this way, i.e., these experiments indicate the possibility of main chain breakdown of DNA by this reaction:



C. *Interference with the Combination with Protamine.* Crosslinked films of the sodium salt of polyacids such as polyvinyl phosphate and

PMA contract (i.e., deswell) when placed in solution of polybases such as polyethyleneimines and protamines, and this was attributed to the formation of a complex held together by salt links, the dissociation of which is prevented by the van der Waals forces between the macromolecules. After some, though not all, of the anionic groups in one of these films had been esterified with the cytotoxic alkylating agents no contraction occurred when these were placed in solutions of polybases and it was thought that the alkylation had prevented combination (Alexander, 1950). This led to the suggestion that reaction of DNA with cytotoxic alkylating agents may interfere with its combination with proteins (Alexander, 1952).

To compare the effectiveness of different compounds in reducing the protein-combining capacity the same treatment could not be used in every case, since the ratio of the amount of alkylating agent that has reacted with DNA to that hydrolyzed varies with the different substances (see p. 24). When conditions were so arranged that approximately equal proportions of the phosphate groups were esterified the results shown in Table III were obtained. The amount of protamine combining with DNA in solution is not constant but depends on the concentration of the reactants and no absolute value can be given (Alexander, 1953); it is, however, possible to obtain comparative values. The affinity of the nucleic acid for protamine was not greatly decreased after reaction with monofunctional compounds during which up to 40% of the phosphate groups had been esterified, whereas a similar reaction with polyfunctional compounds produced a very substantial reduction. In Table III results for four pairs of mono- and polyfunctional compounds are shown in which the degree of esterification was approximately the same. All these polyfunctional substances produce typical "radiomimetic" effects, whereas the monofunctional show no activity in the intact animal.

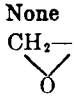
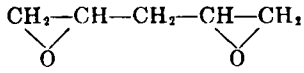
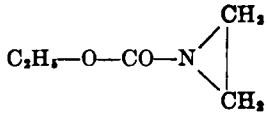
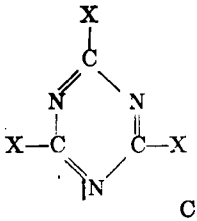
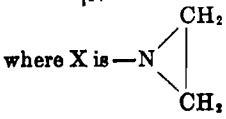
In general, the tendency for intermolecular association is greater between elongated than between coiled macromolecules. A change in shape of the nucleic acid molecule after reaction with a polyfunctional compound which does not occur after a corresponding treatment with a monofunctional compound may explain the observed difference in affinity for protamine.

Possible ways by which mustards may change the shape of the nucleic acid molecule have already been considered and the same factors also apply to the other alkylating agents.

From the protamine-combining studies the suggestion emerged that the change in shape results from reaction of neighboring phosphate groups, as shown in Fig. 8, by reducing the repulsion between charged groups and thereby promoting coiling. The steric restriction imposed on an active com-

TABLE III

Interference with Formation of Nucleoprotein Complex by Alkylating Agents: Differences between Mono- and Polyfunctional Compounds (Alexander, 1952)

Reagent Used	Per Cent Esterification	Ratio of Salmine Combined with Nucleic Acid before and after Reaction
None	0.0	1.0
	25	0.8
	32	0.4
	30	0.8
 where X is 	30	0.5
$CH_3 \cdot SO_2 \cdot O(CH_2)_3 \cdot CH_3$	26	0.9
$CH_3 \cdot SO_2 \cdot O(CH_2)_3 \cdot O \cdot SO_2CH_3$	28	0.4
$(CH_3)_2N \cdot CH_2CH_2Cl$	31	0.7
$(CH_3)N—CH_2 \cdot N(CH_2CH_2Cl)_2$	40	0.2

pound, namely that the reactive groups within the same molecule should be placed in such a way that they can form a ring (see p. 10), follows directly if reaction with adjacent groups is required. It is suggested that the repulsion between charged groups is sufficient to maintain the molecule highly extended even when isolated phosphate groups have been esterified by reaction with a monofunctional compound, but that, after blocking two adjacent anions with a bifunctional compound, the distance separating charged groups is too great for electrostatic effects to keep the molecule rigid at this point. In support of this hypothesis it is known from the

work of Kuhn *et al.* (1948) that the repulsion between the anions in polymethacrylic acid is sufficient to ensure that no significant coiling of the molecule takes place when half the carboxyl groups are un-ionized, but when more of these are blocked so that the distance between charged groups is greater than that between alternate repeating units, the molecule loses its rigidity.

If several two-armed reactions have occurred along the length of the DNA molecule, this may then be far from linear in solution, even though the deviations at any one point may be small. It follows from this hypothesis that a change in the shape of the nucleic acid molecule will only be brought about by monofunctional compounds after extensive reaction, since with excess of phosphate groups it is unlikely that reaction on neighboring sites will occur. This may explain why it may be necessary to use 50–100 times the concentration of a monofunctional derivative to achieve a biological effect comparable with that produced by a polyfunctional compound.

These experiments do not exclude the possibility that the change in shape brought about by the active alkylating agents may be produced by intramolecular crosslinking, but this reaction can only take place if the DNA molecule is highly flexible, whereas the mechanism just discussed could operate, even if the molecule is fairly stiff. Furthermore, the alkylation of neighboring groups requires a special disposition of the active groups very similar to that needed for ring closure which was shown on p. 10 to be a probable criterion for biological activity of the cytotoxic alkylating agents. Such steric restriction would not be operative for crosslinking.

These findings led to the suggestion that the biological effects of the cytotoxic alkylating agents can be attributed to interference with the formation of a nucleoprotein complex or, alternatively, to a change in shape of the macromolecule alone may be sufficient if the suggestion of Haurowitz (1950) is accepted that the role of nucleic acid is to maintain the "template protein" in an expanded state during biosynthesis. The growth-inhibiting activity of stilbamidine is thought by Kopac (1947) to be due to an interference with the combination of nucleic acid with protein, and there may therefore be an unexpected similarity in the mode of action of the amidines as well as of the basic substances (see p. 63) and the cytotoxic alkylating agents.

6. Reaction with Proteins

The crosslinking of proteins by the cytotoxic alkylating agents has already been discussed on p. 16. In this section attention will be confined to the chemical reactions taking place and to the types of groups reacting.

Changes in biological activity such as immunological reactions and enzyme inhibition are beyond the scope of this review.

From general considerations one would expect that at the physiological pH the nucleophilic alkylating agents will react with carboxyl groups, which are largely dissociated, as well as with terminal amino and the imidazole groups of histidine, which are appreciably un-ionized. No reaction would be expected with the ϵ-amino groups of lysine, the guanidinium group of arginine, the phenolic group of tyrosine and the sulfhydryl group of cysteine, all of which exist almost entirely in their unreactive forms at pH 7 (see p. 6).

A. *Mustard Gas.* Following on the discovery of Berenblum and Wormall (1939) that mustard gas altered the immunological properties of proteins, a number investigations have been made of its reactions with a variety of proteins (see review by Boursnel, 1948). From changes in the sulfur content the extent of the reaction with proteins can readily be determined and the salient feature is that the total amount of combination never approaches the value corresponding to reaction with all the available groups (i.e., carboxyl and imidazole).

Herriott (1948) treated the following proteins, all of which reacted with mustard gas, at pH 6: pepsin, egg albumin, chymotrypsinogen, hexokinase, gelatin, serum albumin, serum globulin, fibrinogen, and zein. Detailed analyses of the first four proteins revealed that the carboxyl groups had become esterfied (as shown by titration data) and that only with hexokinase had any reaction with amino groups (Van Slyke nitrogen) taken place. At pH 8, on the other hand, Hartwell (1945) found that after treatment of egg albumin with large amounts of mustard gas part of the protein became insoluble and that between 30 to 70% of the amino groups had reacted. Davies and Ross (1947) showed from titration data that mustard gas could esterify about 20% of the carboxyl groups in serum albumin and horse oxyhemoglobin, and that some reaction had occurred with the imidazole groups. The amino groups were found to titrate at the same pH, but this does not exclude the possibility that reaction had taken place since the pK_2 of a substituted amino group is not necessarily changed (see p. 39). Banks *et al.* (1946) found that the maximum amount of mustard gas which can combine with serum proteins corresponds to an esterification of about 25% of the carboxyl groups present. Young *et al.* (1947) record that zein and gliadin failed to combine, while keratin and salmine reacted with mustard gas. Reaction with salmine is surprising since the only reactive side chains are those of arginine which would not be expected to react at pH 7 to 8; however, the molecular weight of this protein is low so that the total amount of mustard gas combined (55 mg. per gram of protein) could be accounted for by reaction with terminal carboxyl and amino groups.

The esters derived from carboxyl groups in proteins are very labile; thus Alexander *et al.* (1951a) found that esters in wool were hydrolyzed completely in 1 hour at pH 10 and 60°C. and that the rate of saponification was independent of the alcohol used for the esterification in the fourteen cases examined (see also Blackburn *et al.*, 1941). Consequently one would expect mustard gas combined with carboxyl groups to be removed by mild alkaline treatments. Herriott (1948) showed that this was indeed the case and that after two hours at pH 11 and 35°C. the titration curve of pepsin treated with mustard gas reverted to that of the untreated protein (i.e., that all mustard ester groups had been saponified).

Carpenter *et al.* (1948) treated a number of proteins, which had been reacted with radioactive sulfur mustard gas, with alkali and found that the number of residues split off was 70% with pepsin, 30 to 50% with insulin and 86% with the protein moiety of tobacco mosaic virus; in similar experiments with skin proteins (Ormsbee *et al.*, 1949) approximately 50% and with collagen (Pirie, 1946) approximately 70% of the mustard gas combined could be removed with alkali, Pirie (1947) found that the physical properties of collagen were greatly modified by reaction with mustard gas, and in the reviewer's opinion these changes indicate extensive crosslinking. This is supported by the fact that the treated collagen is no longer digested by pepsin. On the other hand, the rate of digestion of kerateine (Peters and Wakelin, 1947) by pepsin was not decreased by mustard gas, and this is in agreement with the finding that mustard gas does not crosslink keratin (Alexander *et al.*, 1952).

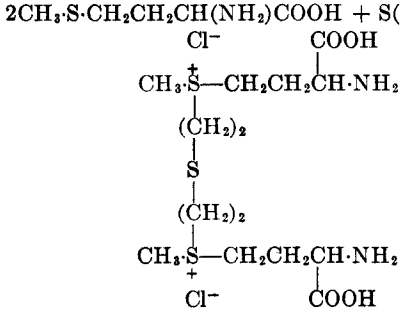
All these investigations indicate that mustard gas combines with carboxyl groups in proteins under physiological conditions, but that only a small proportion are available for reaction; this reaction, however, accounts in general only for about half of the mustard gas combined. There is some evidence that part of the remainder reacts with imidazole groups and reaction with sulfhydryl groups has also been established for serum proteins (Banks *et al.*, 1946), for denatured egg albumin (Hartwell, 1945) and for kerateine (Peters *et al.*, 1947) which is particularly rich in these groups; again, however, only a small proportion of the available groups reacted. Since the pK of protein SH groups is approximately 10 only a very small fraction will be in the reactive (i.e., ionized form) at pH 7, and combination with mustard gas probably takes place because the S^- group has a very high competition factor. Since mustard gas is relatively ineffective in deactivating SH enzymes (Needham, 1948), it would appear that reaction with SH groups is not extensive. Except for the isolated cases of hexokinase (Herriott, 1948) and denatured egg albumin (Hartwell, 1945) no positive results are recorded of reaction with ϵ-amino groups of lysine; also the results of Banks *et al.* (1946), who blocked the amino groups by reaction with isocyanate also indicate that no reaction took place

with these groups in serum proteins. The available evidence therefore suggests that the ε-amino groups do not in general react with mustard gas, but the possibility is not excluded with certainty.

Since the pK of terminal amino groups is considerably lower than that of the ε-amino groups reaction of these with the cytotoxic alkylating agents would be expected and Stevens *et al.* (1948b) found that about 20% of the mustard gas combined with insulin had reacted with the amino groups of phenylalanine, which terminates one of the main chains. A similar reaction could also have occurred in the other proteins studied but may have escaped detection because of their high molecular weight.

Herriott (1948) noticed that a protein after treatment with mustard gas at pH 6 gave less color on subsequent reaction with the Folin phenol reagent, a specific test for tyrosine and tryptophan. The effect was reversed by mild alkaline hydrolysis, and it was tentatively concluded that a labile adduct was formed between the tyrosine hydroxyl groups and mustard gas. This reaction seemed surprising for two reasons: (1), the phenolic hydroxyl groups with a pK greater than 10 are almost entirely in the nonreactive form at pH 6; (2) the ester that could be formed would be stable to alkali. Stevens *et al.* (1948a) showed that the full color value of mustard treated insulin was regained on extraction with sodium dodecylsulfate during which no combined mustard was released. These workers concluded that no reaction had occurred between tyrosine and mustard gas and that the fall in color value was due to an indirect change.

Another possible reaction site in proteins is the thioether group from methionine. The competition factor of sulfur compounds is high (Ogston, 1948) and Stein and Fruton (1946) found that the following reactions with methionine occurred readily:



This compound retains the capacity to alkylate since the sulfonium complex can give rise to a carbonium ion like the original mustard gas. The derivative from methionine is therefore itself a potential cytotoxic alkylating agent. In this way cytotoxic activity may be "stored" in a protein, although there is no evidence that this actually occurs *in vivo*.

The possibility that reaction may take place on the peptide links of the main chain will be considered on p. 40.

Stora *et al.* (1947) found after reaction of mustard gas with gliadin, ovalbumin, serum albumin, oxyhemoglobin, and casein that for each atom of sulfur introduced the proteins contained approximately one atom of chlorine. This led these workers to suggest that only one arm of the mustard gas had reacted, i.e., that the product Protein $—(CH_2)_2—S—(CH_2)_2$ Cl was obtained. Peters and Wakelin (1947) also found chlorine in mustard-treated kerateine, but observed that the amount decreased when the protein was washed for long periods with water. They attribute this to the slow hydrolysis of the chloroalkyl group introduced.

The reviewer feels that a combined chloroalkyl group attached to a protein would not be stable and would hydrolyze during the treatment of the protein with mustard gas and its subsequent extensive washing to remove absorbed mustard gas (e.g., Stora *et al.* washed their proteins for eight days). Kinetic studies with mustard gas and related compounds (cf. Stein *et al.*, 1946) established quite definitely that the rate of hydrolysis of the second chlorine group, i.e., of $R{\cdot}(CH_2)_2{\cdot}S{\cdot}(CH_2)_2Cl$, is fast. A more convincing explanation would seem to be that the "combined" chlorine is present as a "gegen-ion" necessary for maintaining electrical neutrality of the protein after esterification of carboxyl groups. At pH 7 (i.e., not far from the isoelectric point) the majority of the acid and basic groups are internally neutralized and after treatment with mustard gas the chloride ion split off will form a salt with the ammonium ion:

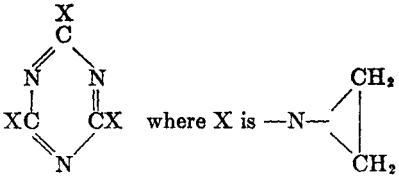
$$\text{Protein—COO}^- \cdots \overset{+}{\text{H}_3\text{N}}\text{—Protein}$$
$$\downarrow +\text{RCl (mustard gas)}$$
$$\text{Protein—COOR} \cdots \overset{+}{\text{H}_3\text{N}}\text{—Protein}$$
$$\text{Cl}^-$$

On extraction with water the chloride ion will exchange slowly with hydroxyl ions and this could account for the "hydrolysis" observed by Peters and Wakelin (1947). This view is supported by the finding that wool after esterification by methyl alcohol using hydrochloric acid as a catalyst contains chlorine which is removed by ion exchange (Alexander *et al.*, 1951a). Also Elmore *et al.* (1948) record that nucleic acids contain no chlorine after reaction with mustard gas, even though with RNA much of the mustard gas combined has reacted with one arm only. No internal neutralization occurs in nucleic acids and no chloride "gegen-ions" are therefore required after esterification.

B. *Nitrogen Mustards, Epoxides, Ethyleneimines and Mesyloxy Compounds.* Fruton *et al.* (1946) found that HN2 reacted with one-quarter and one-third of the amino groups (Van Slyke nitrogen) of egg albumin and

gelatin respectively and a 45% decrease in amino nitrogen (Van Slyke) was observed by Alexander and Fox (1953a) (see Table IV) for serum albumin. An isolated experiment with the aromatic nitrogen mustard derived from *p*-methoxyaniline with wool indicated limited reaction with amino groups (see Table IV).

TABLE IV
Reaction of Cytotoxic Alkylating Agents with Acid and Amino Groups in Serum Albumin and Wool
(Alexander and Fox, 1953a)

Substance Used	Reduction of Serum Albumin[a] in:		Reduction of Wool[b] in:	
	Amino Groups[c]	Carboxyl Groups[d]	Amino Groups[c]	Carboxyl Groups[d]
$CH_3{\cdot}N{\cdot}(CH_2CH_2Cl)_2$	45%	—[f]		
p-$CH_3O{\cdot}C_6H_4N(CH_2CH_2Cl)_2$			21	—[f]
$CH_3{\cdot}CH(O)CH_2$			16	7
$ClCH_2{\cdot}CH(O)CH_2$			47	25
bis—2,3-epoxypropyl ether	49%	15%	47	6
$CH_3{\cdot}SO_2{\cdot}O{\cdot}(CH_2)_4{\cdot}OSO_2{\cdot}CH_3$	31%	0%	8	4
 where X is —N(CH$_2$)$_2$	23%	0%	16	—[f]

[a] A 5% solution of protein was treated at 40% for 72 hours with 3% of reagent except for the ethyleneimine which was used at 0.5% to prevent precipitation of the protein.

[b] 3×10^{-3} mole of reagent were applied per 1 g. of wool suspended in 25 ml. Flask shaken for 72 hours at 40°C.

[c] From Van Slyke determination.

[d] From titration data.

[e] From quantity of dinitrophenyllysine formed on treatment with dinitrofluorobenzene.

[f] Titration data could not be evaluated since introduced basic groups interfere.

Reaction between nitrogen mustards and protein carboxyl groups cannot readily be detected from changes in acid combination since the titration of the mustard amine to its salt and the back titration of the carboxyl ion with acid occur in the same pH range. There is no reason to believe that the nitrogen mustard esters derived from proteins are exceptionally

labile since Davis and Ross (1950) found that the acetates of both aliphatic and aromatic mustards were more stable to acid hydrolyses and only slightly less stable to alkaline hydrolysis than ethyl acetate. Watkins and Wormall's (1952) studies of complement in activation by HN2 while not excluding the reaction with carboxyl groups, indicate that a reaction with another center in the protein also occurs. No experiments have been carried out from which any valid deduction can be drawn concerning the reaction of carboxyl groups with nitrogen mustards; however, the available evidence shows that nitrogen mustards differ significantly from sulfur mustard in that they react with ϵ-amino groups of protein.

Epoxides readily react with amino groups in proteins; Fraenkel-Conrat (1944) found that after treatment with propylene oxide at a pH above 5 the Van Slyke nitrogen was reduced by more than 70% (i.e., 70% of the terminal amino and ϵ-amino groups had reacted). At lower pH values the extent of this reaction was considerably less. These results were confirmed by Alexander and Fox (1953a) for three epoxides with wool and for a bis-epoxide with serum albumin (see Table IV). The combination of an epoxide with an amino group, i.e.,

$$\text{Protein—NH}_2 + \text{CH}_2\text{—CHR} \rightarrow \text{Protein—NH·CH(OH)·CH}_2\text{R}$$

does not suppress their basic nature and the pK is hardly changed. For this reason it is impossible to detect reaction of epoxides with amino groups from titration data, and this probably also applies to combination with the other cytotoxic alkylating agents.

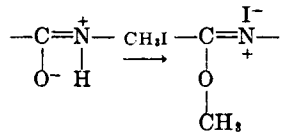
Fraenkel-Conrat (1944) finds that the color obtained with the Folin phenol reagent on intact proteins is reduced after reaction with epoxides and deduces that extensive reaction with phenol and indole groups has occurred. This test, however, is suspect (see p. 36) as it was shown that a similar reduction in color with mustard gas-treated proteins could be reversed without liberating any mustard groups. Although a similar experiment was not carried out with epoxide-treated proteins, the possibility that combination with amino acid residues other than tyrosine and tryptophan may be responsible for the decreased color cannot be discounted.

Evidence for reaction of epoxides with carboxyl groups at or near pH 7 is conflicting. The carboxyl groups in proteins back titrate with acid in the presence of salt between pH 5.5 to 2.5 and esterification can thus be determined by measuring the decrease in acid uptake which occurs in this pH range. Using this method Alexander *et al.* (1951a) found that of the six epoxides studied only epichlorhydrin reacted with more than 10% of

the carboxyl groups present in wool (see also Table IV). Similarly only 15% of the carboxyl groups of serum albumin reacted with a bis-epoxide (see Table IV). Contrary to these results Fraenkel-Conrat (1944) claims that with propylene oxide more than 50% of the carboxyl group of egg albumin and β-lactoglobulin were esterified. The method of estimation consisted of a determination of the amount of basic dye which combines with the protein at pH 11.5 after standing for at least 24 hours. There is ample evidence to show that the ester groups in proteins are completely saponified under these conditions (see p. 35), and this method of analysis is therefore useless for determining esterification of carboxyl groups. This objection was appreciated by Fraenkel-Conrat some years later when he and Olcott (1946) showed that the dye uptake method could not be used for following esterification of proteins by alcohols. The shift in the isoelectric point of egg albumin from pH 5 to 8 on treatment with propylene oxide noted by Fraenkel-Conrat (1944) could result from the blocking of approximately 10% of the carboxyl groups and could also have been brought about by the quaternization of the imidazole group of histidine, which was shown to occur with mustard gas (Davies and Ross, 1947); it does not therefore provide confirmatory evidence for extensive reaction with the carboxyl groups. The limited amount of valid data indicates that epoxides in general do not react extensively with carboxyl groups of proteins near their isoelectric point except for epichlorhydrin, which can react with up to 40% of the carboxyl groups in wool.

Only preliminary studies have been made of the reaction of ethyleneimines and mesyloxy compounds with proteins and the results are shown in Table IV. These alkylating agents do not appear to esterify carboxyl groups, but in every case react with amino groups. These groups are also involved in the crosslinks formed in wool since fibers in which the amino groups have been blocked by acetylation cannot be crosslinked with epoxides or mesyloxy compounds.

C. *Main Chain Degradation.* Reaction of alkylating agents with the peptide bond is not impossible and Blackburn *et al.* (1941) showed that the number of methyl groups introduced into wool and silk by methyliodide and dimethylsulfate is greater than the number of carboxyl groups present. The possibility that enolization of the peptide bond occurs in acid solutions of proteins has frequently been envisaged and was proved for the polyamide nylon from acid combining data (Carlene *et al.*, 1947). At neutrality such a reaction is not likely to occur, but there is evidence that diketopiperazine exists in part as a resonance hybrid containing a zwitterionic peptide link (Corey, 1938) and Blackburn *et al.* (1941) envisage the possibility that some peptide links may react with alkylating agents via the following zwitterionic form.



Such a structure would not be stable and may hydrolyze in such a way as to bring about peptide bond fission.

An indirect indication that a cytotoxic alkylating agent degrades proteins came from the discovery that serum protein after incubation with mustard gas increased capillary permeability. Cullumbine and Rydon (1946), suggested that a polypeptide had been liberated since earlier workers had found a factor capable of inducing capillary permeability in enzymatically digested proteins and had established that the active prin-

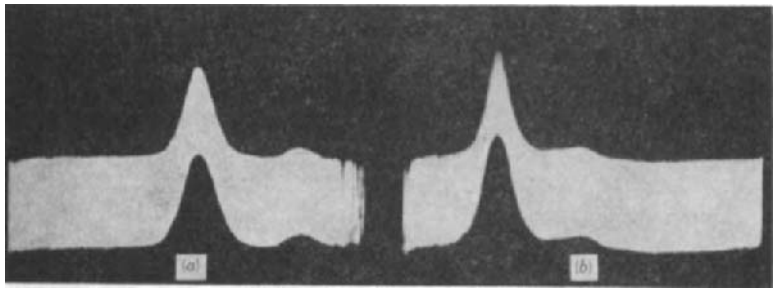


FIG. 9. Electrophoretic pattern of serum albumin after treatment with bifunctional alkylating agents: (a) serum albumin treated with HN2; (b) serum albumin treated with $O(CH_2CH—CH_2)_2$.

ciple named leukotaxine was a polypeptide of relatively low molecular weight. Further evidence for degradation of proteins by sulfur and nitrogen mustards and epoxides was obtained by electrophoretic examination of the treated protein which were seen to consist of two compounds, both having different mobilities from the starting material; with HN2 the second component was approximately 6.5% of the total and with bis-(2,3-epoxypropyl) ether 8.5% (see Fig. 9).

D. *Summary.* The reactions of the carboxyl of proteins near their isoelectric point with the cytotoxic alkylating agents is anomalous. From chemical consideration, supported by experiments with model substances and other macromolecules (see p. 17), reaction with the carboxyl groups, and no reaction with the amino groups is to be expected. Mustard gas comes closest to these predictions, but even with this compound the fact that at the most half of the available carboxyl groups react is surprising. The ready reaction of the amino groups with nitrogen mustards, epoxides,

and possibly also with the ethyleneimines and mesyloxy compounds is completely contrary to expectation. This anomaly may be due to the fact that these groups are hidden within the protein and sterically inaccessible to the alkylating agents, but this does not seem likely. Alexander *et al.* (1952c) suggested that the chemical reactivity of the internally neutralized carboxyl and amino groups was not correctly represented by $-COO^- \cdots H_3^+N-$ but that they behaved in chemical reactions as $-COOH \cdots H_2N-$. This suggestion is supported by the finding (Rutherford *et al.*, 1940) that isoelectric proteins are esterified by diazomethane which can react only with unionized carboxyl groups. The observation of Banks *et al.* (1946) that the amount of mustard gas that can combine with serum proteins is doubled if the amino groups have been reacted with isocyanate can also be interpreted according to this hypothesis, since after blocking the amino group the corresponding carboxyl groups will no longer be restrained as zwitterions and can then react with mustard gas at pH 7.

III. Ionizing Radiations

An extensive literature (cf. Haddow, 1953) has accumulated on the ability of x-rays, as well as α-, β-, and γ-rays and neutrons to induce tumors. These radiations inactivate enzymes and viruses and have been shown to degrade, to crosslink, and to aggregate macromolecules. The primary reaction of all these radiations on hitting a molecule is one of ionization (i.e., ejection of an electron). There are, however, two distinct mechanisms by which they can modify a macromolecule; if this is irradiated dry or in very concentrated solution, the action is directly on the macromolecule, which is ionized and in this highly reactive form can undergo different secondary reactions. If the macromolecule is present in dilute solution (in this review aqueous solutions only will be considered), the primary reaction is with the solvent, which breaks up into radicals, and these can then react with the macromolecule. In relatively concentrated solutions or in gels, such as may exist in cells, both reactions may be operative simultaneously.

In its simplest form direct action can only produce a change if the ionization occurs within the sensitive area of an organism or a macromolecule. The area of the "target" may, however, be considerably extended if the reaction of the ejected electron is considered. Thus after a direct hit of a macromolecule (M) the following series of reactions may occur in a living system (Burton, 1952).

$$M \xrightarrow[\text{radiation}]{\text{ionizing}} M^+ + e$$

$$e + H_2O \rightarrow H + OH^-$$

$$H + O_2 \rightarrow HO_2$$

Thus even if the ionization itself does not lead to a biological effect because it has not occurred in a sensitive region (i.e., not within the target) the HO_2 radical produced may diffuse to the target site and react there. Considerations such as these may resolve the controversy (cf. Gray, 1952) whether the action of radiations on living cells is direct or via free radicals.

In very dilute solutions it is improbable that an ionization of the macromolecule will occur and the ionization products of water have to be considered as the active intermediaries. The radiolysis of water is not adequately understood and there is no general agreement concerning the radicals formed (cf. *Faraday Society Discussion*, 1952).

The primary reactions which are generally accepted are

$$\begin{array}{l} H_2O \rightarrow H_2O^+ + e \\ H_2O^+ \rightarrow OH + H^+ \\ \hline H_2O \rightarrow OH + H^+ + e \end{array}$$

and there is abundant evidence for the existence of the reactive OH radical. The reaction of the low-energy ejected electron (the thermal electron) is generally thought to result in the formation of H atoms by a reaction which may be of this type.

$$H_2O + e \rightarrow H + OH^-$$

It should, however, be stressed that there is no direct experimental evidence for an H atom and a number of experiments (cf. Dainton, 1952) contraindicate its formation in this way. Recombination of radicals leads to the formation of hydrogen peroxide and in the presence of oxygen (e.g., dissolved air) the radical HO_2, which is more persistent than OH (Burton, 1952), is also formed. In aerated aqueous solutions therefore the effect of ionizing radiations is to bring about the formation of OH, HO_2 radicals, and possibly H atoms, as well as H_2O_2. In general, irradiation of water can be considered as the introduction of a powerful oxidizing agent.

Whether a given effect of radiation is due to direct action (i.e., target effect) or due to radicals produced in the solvent medium can often be determined by applying two tests. First, if the proportion of the substrate altered (e.g., inactivated) is independent of concentration, the action of the radiations must be direct; if, on the other hand, the total amount of change is independent of concentration (i.e., the proportion changed decreases with increasing concentration), the reaction is produced by radicals obtained from the solvent. Secondly, if the amount of change of the macromolecule is decreased by the presence of another solute (i.e., a protective effect), the action of the radiations is almost certainly indirect and the two substrates compete for a limited number of

radicals. Many effects of radiations are potentiated by the presence of oxygen, and this is often considered as evidence for indirect action, the oxygen bringing about the formation of the persistent radical HO_2 and hydrogen peroxide. The possibility that oxygen sensitizes the substrate and thereby increases the target area cannot, however, be neglected for some reactions, such as the breaking of chromosomes.

1. Reactions of Proteins

A. *Loss of Biological Activity.* After irradiation with different ionizing radiations almost all proteins lose their specific biological properties such as enzymatic, virus, or immunological activity. This occurs in general both when the proteins are irradiated dry or in aqueous solution. Analyses of the results obtained with dry preparations led Lea to develop the target theory which cannot be discussed here (cf. Lea, 1946). In a review of the work done before 1939 Fricke (1938) stresses that in aqueous solution the inactivation of proteins is due to a reaction with "activated water" and not due to direct ionization, and this view has been fully substantiated by the subsequent work of Dale (summarized, 1952), who showed that a number of simple substances such as thiourea and sodium formate were capable of protecting dilute solutions of crystalline enzymes. The significance of much of the earlier work is limited because of the impurity of the protein preparations used and only the work of Northrop (1934) with crystalline pepsin is uncomplicated in this respect. The sensitivity of different proteins to deactivation by ionizing radiations varies widely, and this aspect is reviewed by Fricke (1938) and Arnow (1936). An important observa-

TABLE V
Continued Inactivation of Trypsin Solutions after Cessation of Irradiation
(MacDonald, 1953)

	Per Cent Original Activity Left			
	2°C.		25°C.	
Hours	Control	Irradiated	Control	Irradiated
0	100	49	100	49
1	100	49	103	49
3	103	50	101	47
8			101	45
18			98	40
24	98	50	98	37
48	99	47	99	29

Note. Concentrations of trypsin, 0.04 mg. per ml.; solvent, 0.005 *N* sulfuric acid; dosage of X-rays 2500 r.

tion was recently made by MacDonald (1953) and Anderson (1953), who found that trypsin and pepsin respectively continued to decrease in enzymatic activity after irradiation was complete (see Table V). This after effect is very temperature dependent and differs in this respect from the degradation of nucleic acid, which also continues after irradiation (see p. 48).

B. *Physical and Chemical Changes.* Relatively little is known concerning the actual chemical reactions which occur. Fricke (1938) found that one of the products is hydrogen gas and concluded that the "activated water," or as we now know the free radicals, bring about an oxidation. The most obvious physical effect with all proteins is denaturation, which may result in coagulation or render the protein more easily coagulated by heat, but the chemical basis of these changes is not known. Other physical effects produced are changes in optical rotation, refractive index, surface tension, and electrical conductivity (see Arnow, 1936).

Astbury and Woods (1933) found that the mechanical properties of wool fibers were extensively modified by irradiation with x-rays and the most probable interpretation of these changes seems to be that both disulfide crosslinks and main chain peptide bonds are broken. Further work along these lines should be carried out since information on changes in fiber properties produced may advance the understanding of the mechanism of the breaking of chromosomes by ionizing radiations.

The absorption spectrum of proteins is changed by irradiation though some of the earlier workers differed as to whether the characteristic band at 2800 A. was increased or decreased in intensity (see Arnow, 1936). Barron (1952) has studied the problem in detail and found that x-rays increased the absorption (see Fig. 10) and that the effect of a dose as small as 100 r. could be detected. The increase is greatest when the protein is irradiated near its isoelectric point. Arnow (1935) suggested that the increased absorption may be due to oxidation of phenylalanine residues to tyrosine, and this suggestion is in agreement with the more recent work on the oxidation reactions of the free radicals produced in water and is also supported by the finding of Barron (1952) that the increase in absorption is greater if the irradiation is carried out in the presence of oxygen.

Ionizing radiations change the viscosity of protein solutions; with gelatin a gradual decrease is observed after irradiation with x-rays (Sokolov, 1940), and this effect can probably be ascribed to degradation resulting in a shortening of the protein chain, but with globular proteins the effect is more complicated. Arnow (1935) found that the viscosity of egg albumin solutions exposed to α-particles is increased if the protein solution is at or below its isoelectric point, but decreased at higher pH values. The evidence is not sufficient to decide whether the increase is due to aggregation or due to an opening of the globular molecules giving rise to

a more asymmetric structure. The decrease can probably be ascribed either to degradation leading to a smaller molecule or to a change in hydration. Svedberg and Brohult (1939) found that α-rays degrade hemocyanin and serum albumin. The reaction with the former is particularly interesting since the giant molecule is split either into halves or eighth and the effect of the radiations is to break up a highly specific structure into

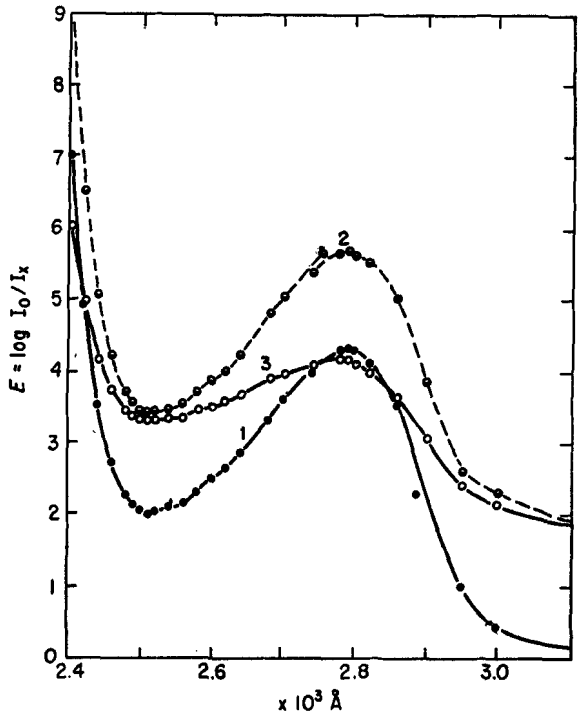


FIG. 10. Effect of irradiation with x-rays on the absorption spectrum of bovine serum albumen (10^{-5} M) (Barron, 1951): (1) not irradiated; (2) irradiated with 5×10^4 r.; (3) irradiated with 10^5 r.

smaller and more stable units. The process here is not random as in the degradation of synthetic macromolecules. Recent papers have appeared on the action of x-rays on dry fibrinogen (Koenig and Perrin, 1952) and fibrinogen in aqueous solution (Sheraga and Nims, 1952); in both cases an increase in viscosity was found and studies in the ultracentrifuge showed that a component of higher molecular weight appeared. The most probable explanation here, is aggregation of the protein molecules possibly by oxidation of sulfhydryl groups. The observation that the change in fibrinogen in aqueous solution can be reduced by protective agents established that the reaction is due to free radicals.

In few cases is there any evidence which indicates which of the radicals produced in water are responsible for the reaction. In most of the experiments air was not excluded so that besides the OH radicals HO_2 radicals and hydrogen peroxide were also present and where the presence of air leads to an enhancement of the effect these may be involved. The effect of oxygen may, however, also be due to the formation of unstable peroxides (see p. 55). In a few cases, however, such as the inactivation of carboxypepsidase and ribonuclease (Dainton and Holmes, 1950) with x-rays the reaction is independent of oxygen and can therefore be attributed to OH radicals. If the reaction were due to hydrogen atoms less reaction would be expected in oxygen which competes for them.

A chemical reaction produced by ionizing radiations which has been established on a detailed quantitative basis (Dale *et al.*, 1949) is the deamination of amino acids with liberation of ammonia. The importance of this reaction in proteins has not yet been established nor is it known if it contributes to their loss of biological activity. A reaction to which much attention has been focused by Barron and his colleagues (summarized Barron, 1952) is the oxidation of sulfhydryl groups. Barron found that enzymes which require sulfhydryl groups for activity such as phosphoglyceraldehyde dehydrogenase, adenosine triphosphatase, and succinodehydrogenase were all inactivated by x-rays with an ionic yield which was higher than that obtained for inactivation of nonthiol enzymes. Moreover, after low doses of x-rays the enzymes could be completely reactivated by treatment with glutathione which reduces oxidized thiols. The reactions which according to Barron can take place in oxygenated solutions are:

$$2 - SH + 2OH = —S—S— + 2H_2O$$
$$2 - SH + 2HO_2 = —S—S— + 2H_2O_2$$
$$2 - SH + H_2O_2 = —S—S— + 2H_2O$$

and from a detailed study with glutathione OH radicals were shown to contribute 23%, HO_2 43%, and H_2O_2 24% of the total oxidation. Dale and Davies (1951) found that oxidation of —SH to —S—S— was not the only reaction which occurs on irradiating cysteine and glutathione and that under certain conditions hydrogen sulfide is split off and this may explain why Barron found that with larger doses of x-rays a proportion of the thiol enzymes were inactivated irreversibly. Whether the inactivation of SH enzymes by oxidation is biologically significant has not, however, been established. In general the reducing capacity of a living organism is such that it could readily cope with the total amount of oxidation brought about even by very large doses of ionizing radiations and since Barron has shown the ready reversibility *in vitro* this would be expected to occur automatically *in vivo*.

2. *Reactions of Deoxyribonucleic Acids (DNA)*

A. *Depolymerization.* Sparrow and Rosenfeld (1946) found that the viscosity and streaming birefringence of high molecular weight DNA in dilute aqueous solutions was reduced after irradiation with x-rays and that log (decrease in viscosity) was proportional to the dose of irradiation. In the presence of histone and molar sodium chloride the sensitivity to

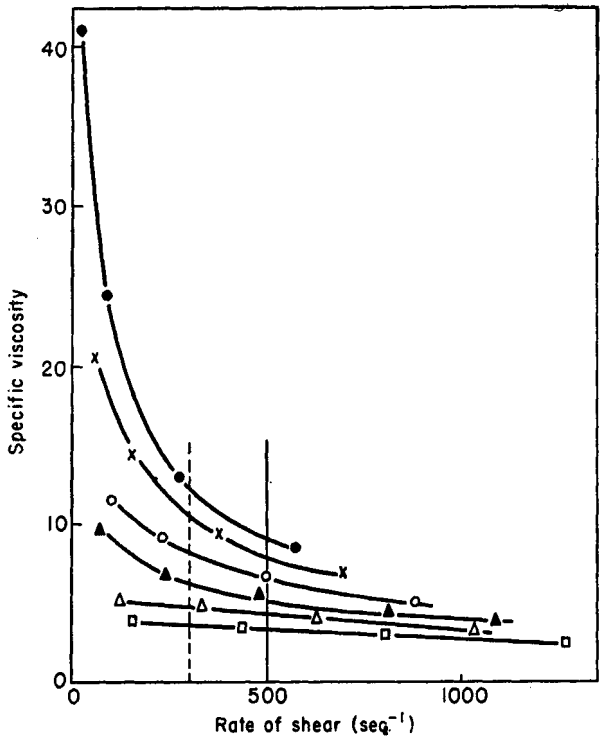


FIG. 11. Effect of irradiation with x-rays on viscosity of deoxyribose nucleic acid (0.3%) (Taylor *et al.*, 1948):

- ● not irradiated
- × irradiated with 5,600 r.
- ○ irradiated with 11,200 r.
- ▲ irradiated with 16,800 r.
- △ irradiated with 22,400 r.
- □ irradiated with 28,000 r.

x-rays was decreased. The authors conclude that the length of the DNA molecule was reduced by x-rays as a result of depolymerization. This degradation was studied in more detail by Taylor *et al.* (1948), and their results are shown in Fig. 11. They further made the most significant observation that the decrease in viscosity continued after the irradiation was stopped and the magnitude of this after effect (Fig. 12) was only slightly dependent on temperature. They found that the presence of serum albumin reduced the initial effect of the x-rays, but did not influence the

after effect. Other substances such as thiourea (Limperos and Mosher, 1950), cyanide, and β-mercaptoethylamine (Conway and Butler, 1953) decreased both the initial and the delayed decrease in viscosity. It is important that none of these protective agents was capable of preventing the slow after effects if added after the irradiation and they must function by competing for the degrading radicals and not by combining with the unstable form of DNA, which slowly decomposes. From ultracentrifuge

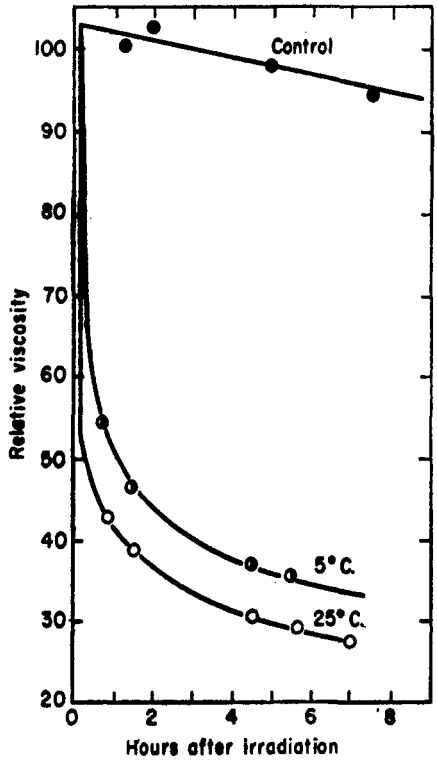


FIG. 12. Decrease in viscosity of deoxyribose nucleic acid after irradiation with x-rays (56,000 r.) has been finished. Effect of temperature on the after effect is shown (Taylor *et al.*, 1948).

studies Taylor *et al.* (1948) concluded that the x-rays depolymerized the DNA and gave a polydispersed product of lower molecular weight (see also Limperos and Mosher, 1950; Conway *et al.*, 1950).

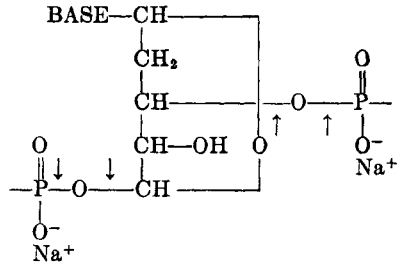
In a detailed investigation of the after effect, Butler and Conway (1950) found that if DNA is irradiated in the complete absence of oxygen there is a small initial decrease in viscosity but no after effect. It is reasonable to suggest that the initial degradation is due to OH radicals, but the reactions leading to the after effect are more complicated. Thus, although pure samples of DNA are not degraded by hydrogen peroxide, if this is

added to DNA after irradiation in the absence of oxygen a slow depolymerization, similar to that occurring after irradiation in oxygen, takes place. These experiments suggest that there may be some latent damage by OH radicals which renders DNA more susceptible to hydrogen peroxide. It is interesting that bacteriophage also are more sensitive to hydrogen peroxide after irradiation with x-rays (Alper, 1953). The quantity of hydrogen peroxide formed, however, by irradiation of water is not sufficient to account for the whole of the after effect observed for DNA. Conway and Butler (1952) suggest as a contributory cause that the HO_2 radical formed only in the presence of oxygen reacts with DNA to form a peroxidic derivative which undergoes a further slow change resulting in degradation and contributes to the observed after effect. Although this is by no means the only mechanism compatible with the available data it is supported by the finding (Alexander and Fox, 1952b) that the chemicals which, if present during the irradiation, prevent the after effect, react readily with HO_2 radicals, and can combine with these competitively.

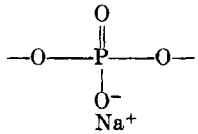
Limperos and Mosher (1950) found that DNA extracted from x-irradiated rats had a significantly lower viscosity than that from control animals, but the dose required to produce this degradation *in vivo* was only about one-tenth of that necessary to produce a similar viscosity change *in vitro*. Thiourea prevents both the *in vivo* and *in vitro* degradation. A possible explanation for the increased sensitivity *in vivo* was advanced by Mosher (1950), who suggested that there may be a phosphatase enzyme which degrades DNA but which can act only via secondary phosphate groups. Normally the action of the enzyme is slow since there are only relatively few secondary phosphate groups in DNA as these can only occur at the end of the long molecules. However, on irradiation more secondary phosphate groups are formed and the effect of the x-rays is then multiplied *in vivo* by this enzyme.

In an interesting study, which is difficult to interpret in chemical terms, Errera (1947) found that the rigidity of a nucleoprotein gel extracted from nuclei of chicken erythrocytes was significantly reduced after irradiation with x-rays. A smaller though still significant decrease occurred if the whole cell was irradiated and the rigidity of the nucleoprotein gel extracted afterward was measured. The gel structure is intimately related to DNA and the loss in rigidity can probably be attributed to degradation of this molecule. If an insoluble nucleohistone is suspended in water and then irradiated (Rollenaal *et al.*, 1951), dissociation of the complex occurs and some of the DNA goes into solution. A possible interpretation is that the affinity of DNA for histone is reduced on irradiation, as it is after treatment with the cytotoxic alkylating agents (see p. 31).

B. *Chemical Changes.* To bring about depolymerization of DNA bonds forming the main chain have to be attacked and only reaction at the points indicated by an arrow would lead directly to degradation



Reaction with the purine or pyrimidine bases, or with the sugar ring would not decrease the molecular size except by initiating further reactions such as hydrolysis. With a polyphosphate of the following constitu-



tion having a molecular weight of the order of 2×10^6 no degradation could be detected after irradiation in aqueous solution with x-rays at doses of 10,000 r. (Alexander and Fox, 1953b). Since under comparable conditions polyvinyl compounds (see p. 54) are substantially reduced in molecular weight it would appear that the O—P bond is very resistant to radiation and that reaction occurs much more readily at carbon bonds. These experiments with model substances indicate that a likely point for x-rays degradation of DNA is the glycoside O—C bond. This suggestion is supported by the observations that polysaccharides are decomposed by x-rays with the liberation of organic acids (Khenokh, 1950) and that one of the first chemical changes which can be detected in cells after irradiation of DNA is a reduction in the staining reaction due to sugars (Claude, private communication).

Taylor *et al.* (1948) found after an irradiation, sufficient to reduce the molecular weight of DNA to less than half, that no inorganic phosphate, ammonia, or dialyzable nucleotide residues were split off. The enzymatic susceptibility also was unchanged as was the extinction coefficient at 2600 A. More recently Barron (1952) has found very minor changes in the absorption spectrum of DNA after irradiation. However, if aqueous solutions of DNA and RNA are treated with extremely large doses of x-rays (1 to 4×10^6 r.) extensive chemical changes can be detected (summarized Scholes and Weiss, 1952), such as the formation of ammonia, inorganic phosphate, and free purine and pyrimidine bases. Almost every

part of the nucleic acid molecule seems to be susceptible to attack and deamination and ring opening of the heterocyclic bases, fission of glycoside linkages with liberation of purines, breaking of the ester link with the formation of inorganic phosphate, and an increase in titratable groups have been established. These experiments, however, do not throw any light on the nature of the depolymerization which occurs at much lower doses. Scholes and Weiss (1952) have found some evidence that the after effect with DNA is due to the formation of labile ester groups. The formation of ammonia is favored by the presence of oxygen and it is concluded that HO_2 radicals play a part in this reaction. The liberation of inorganic phosphate is not, however, increased by the presence of oxygen, and it is therefore thought that OH radicals only are involved.

C. *Reaction with Chemically Produced Free Radicals.* OH and HO_2 radicals can be produced in water by chemical reactions. One way is to irradiate solutions of hydrogen peroxide or substituted peroxides such as *t*-butyl peroxide with ultraviolet light when the main photochemical reaction is $H_2O_2 \xrightarrow{UV} 2OH$. HO_2 radicals are formed simultaneously according to the reaction $OH + H_2O_2 \rightarrow H_2O + HO_2$. When ferrous sulfate is oxidized by hydrogen peroxide, both OH and HO_2 radicals are produced during the reaction (Haber and Weiss, 1934; Abel, 1948), and these are responsible for the strong oxidizing properties of Fenton's reagent (i.e., a mixture of ferrous sulfate and hydrogen peroxide). OH and HO_2 radicals are now known (Bacon, 1946) to be formed in many other redox systems involving an electron transfer reaction (e.g., ascorbic acid and hydrogen peroxide). Weiss, Scholes, and Stein (1949) found that chemically produced free radicals degrade DNA and breakdown products similar to those obtained with x-rays were formed. Butler, Gilbert, and Smith (1950), Smith *et al.* (1951), and Limperos and Mosher (1950) independently recorded that chemically produced free radicals produced by mixed redox systems and by the photochemical decomposition of peroxides reduce the viscosity of DNA solutions by breaking the main chains. The physical changes were very similar to those found after irradiation with x-rays, and these experiments provide additional support for the suggestion that the degradation by x-rays is due to OH and HO_2 radicals. Smith *et al.* (1950) record that methanol and glucose act as protective agents in the degradation of DNA by photochemically produced free radicals, and this indicates that HO_2 radicals play a part as these substances also protect the degradation of polymethacrylic acid which is thought to be due to these radicals (see p. 55).

3. Effect on Synthetic Polymers

Within recent years a large amount of work has been done on the behavior of plastics when exposed to radiation from an atomic pile.

Sisman and Bopp (1951) examined the change of mechanical properties of thirty-three different plastics, the radiation resistance of which they place in the order shown: phenol-formaldehyde, styrene, aniline-formaldehyde, polyethylene, nylon, polyesters, urea-formaldehyde, vinyl chloride and acetate, casein, methylmethacrylate, polytetrafluoroethylene cellulose derivatives. This order can be considered as a rough guide only since deterioration was assessed by different tests the results of which do not always run parallel. It is interesting that this order is not the one in which these materials would be placed with regard to general inertness to chemical and photochemical attack. Thus vinyl polymers and particularly polytetrafluoroethylene are more resistant to chemical attack than the phenol-formaldehyde resins. Little (1952) distinguishes between exposure to the pile in the presence and absence of oxygen; in the first case deterioration generally sets in, whereas in the second case the material may often be strengthened. Degradation appears to occur more readily in the noncrystalline parts of the polymers and the x-ray diffraction patterns are sometimes unchanged even though the mechanical properties have been completely altered. Little believes that this is due to the fact that the crystalline micelles have not been changed by the radiation. This, however, is not a necessary conclusion as it is known from studies with textile fibers that the x-ray diffraction pattern remains unchanged even after most extensive chemical changes have taken place within the micelles. Charlesby (1952) exposed polyethylene to neutrons and γ-rays and established that crosslinking between the chains occurs. The extent of this reaction was found to be proportional to the dose and with a pile dose of 10^{17} slow neutrons per square centimeter approximately 1% of all the carbon atoms take part in crosslinks. Alkyl radicals and hydrogen atoms are thought to be formed, but only the former bring about crosslinking. The hydrogen atoms are not thought to react since their small size allows them to diffuse away from the site of the ionization. A general interpretation is that there are two distinct reactions when polymers are irradiated, degradation or crosslinking. Although on absorbing ionizing radiations the quantum of energy is greatly in excess of that required to break main chain carbon bonds, these will immediately recombine again because of the close proximity in which the resulting free radicals find themselves in a solid or liquid. Decomposition therefore only occurs when a molecular rearrangement into two stable entities which can not recombine is possible. This is known as the "cage effect." Hydrogen atoms because of their small size can diffuse away and reactive centers are thus formed which can combine to give crosslinking. Thus polyethylene which cannot rearrange along its main chain to give stable structures crosslinks while acrylates which can rearrange by splitting off CO_2 degrade.

The degradation of high polymers in solution is of greater significance

to the understanding of the biological effects than the results on the solid plastics reported above. However, only polymethacrylic acid (PMA) in aqueous solution has been studied in detail (for the results of irradiation of solutions of a synthetic polyphosphate see p. 51). PMA is readily degraded on irradiation with x-rays, and low doses (e.g., 100 r.) can be detected if a high molecular weight polymer is used in very dilute solutions (Alexander and Fox, 1952a). The change in viscosity of PMA solu-

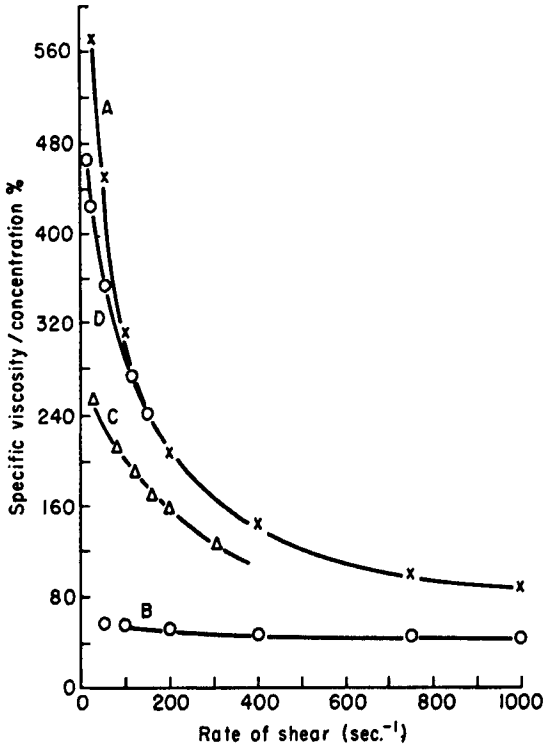
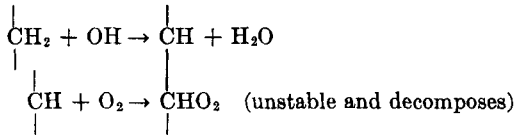


FIG. 13. Effect of 400-kv. x-rays on viscosity of 60% neutralized high molecular weight polymethacrylic acid (Alexander and Fox, 1952): A, 0.08% polymethacrylic acid; B, 0.08% polymethacrilic acid subjected to 4000 r.; C, 0.025% polymethacrylic acid subjected to 200 r.; D, 0.025% polymethacrylic acid subjected to 50 r.

tions on irradiation is seen in Fig. 13, and this was shown to be due to breakdown of the molecule by unambiguous molecular weight determinations (e.g., irradiation of a 0.14% solution of PMA with 6000 r. reduced the molecular weight from 1.1×10^6 to 2.6×10^5). The number of carbon-carbon bonds broken per unit weight of polymer is directly proportional to the x-ray dose and inversely proportional to the concentration of the polymer in solution (Alexander and Fox, 1953b), and consequently the degradation must be due to the free radicals formed in the water.

No direct action of the x-rays can be detected under the conditions of the experiment. No change in viscosity was observed when the irradiation was carried out in the absence of dissolved oxygen. The degradation cannot therefore be caused by OH radicals or H atoms, but is thought to be due to reaction with HO_2 radicals which are formed in the presence of oxygen. Hydrogen peroxide, another product of irradiation of aerated water, does not degrade PMA by itself (i.e., in the dark). The possibility that the decomposition of an unstable peroxide formed by the successive reaction of an OH radical and molecular oxygen, thus:



leads to polymer breakdown was eliminated by experiments with hydrogen peroxide which has a potentiating effect. No after effect as has been observed with nucleic acid could be detected with PMA. Free radicals produced by the photochemical decomposition of hydrogen peroxide and with Fenton's reagent (see p. 52) degrade PMA as do x-rays.

The degradation of PMA could be prevented by the addition of amines, thiourea, cyanide, and other substances which were known to decrease the mortality of mammals when exposed to lethal doses of radiation. These substances protect PMA by competing for the HO_2 radicals since many of the protective agents do not appear to compete effectively for OH radicals. Alexander and Fox (1952b, 1953b) conclude that radiation sickness and delayed death (i.e., radiation effects against which these substances protect) result from the reaction of HO_2 radicals. Effects such as the breaking of chromosomes against which they do not protect (Devik, 1952) may be due to other radicals or to direct action. It would be interesting to see if the incidence of x-ray-produced tumors was affected by the administration of protective agents.

IV. Polycyclic Hydrocarbons

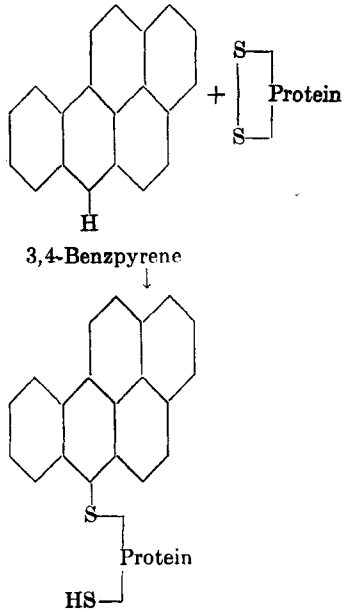
A member of this group of compounds was the first pure chemical substance to be identified as a carcinogen. In the last twenty years the chemistry of the carcinogenic hydrocarbons has been studied most intensively, yet their mode of action is not understood (cf. Haddow, 1953). These compounds are readily changed in the body, and many of the metabolic products are now known; but since none of these have proved biologically active, their reactions will not be discussed here and attention will be confined to the unchanged hydrocarbons. In spite of the large body of research which has accumulated concerning the chemistry of

these hydrocarbons, only a few investigations have been reported of their interaction with macromolecules.

1. Combination with Tissue Constituents

Following on the demonstration by Heidelberger and Jones (1948), using radioactive tracers, that a part of the carcinogenic hydrocarbons remains at the site of application for many months, Miller (1951) showed that 3,4-benzpyrene painted on mice combined with proteins in the epidermis though not with proteins in other parts. The firm combination which takes place rapidly (i.e., within a few hours) must be the result of a metabolic reaction in the skin since no protein bound hydrocarbon was found if freshly killed mice were painted. Although the nature of the linkage between the hydrocarbon and the protein is not known, the fact that it is not split by extraction or by dissolution of the proteins followed by reprecipitation indicates strongly that it is not adsorption but that covalent bonds are involved. As an extension of this work Heidelberger and Weiss (1951) found that, after intravenous injection of radiolabeled 3,4-benzpyrene and 1,2,5,6-dibenzanthracene, part of the radioactivity became associated with proteins, indicating that combination had occurred with the hydrocarbon or their metabolites.

Ten years before the *in vivo* combination of hydrocarbons with proteins was demonstrated Fieser (1941) put forward the suggestion that the carcinogen combined with cell proteins by the opening of an —S—S— linkage, e.g.,



Such a reaction was demonstrated *in vitro* with chlormethyl benzanthracenes and cysteine (Wood and Fieser, 1940), though no reaction would be found with the unsubstituted hydrocarbon. Crabtree (1944, 1945) approached the problem differently. He showed that substances which react with SH compounds, if applied before the carcinogenic hydrocarbons, delay the time of appearance of tumors. The substances used were acid chlorides and unsaturated dibasic acids; their relative effectiveness in impeding carcinogenesis paralleled their rate of reaction with cysteine. Another class of active inhibitors consists of compounds such as bromobenzene which are known to react in the body with SH compounds. A possible interpretation of these experiments is that an essential step in the carcinogenic action of the hydrocarbons is combination with SH groups, which are blocked by the inhibitors. The observation that prior application of SH compounds does not retard carcinogenic action (Crabtree, 1948) would indicate combination of the hydrocarbons occurs only with certain tissue SH groups and that extraneous compounds do not compete with this reaction. Reimann and Hall (1936) found a reduction in tumor incidence if thiocresol was painted for several weeks prior to the first application of hydrocarbon. This observation cannot be considered to contradict Crabtree's findings since the prolonged painting produced pronounced histological changes.

Combination with proteins *in vitro* has not been satisfactorily demonstrated. The only evidence for it (Wunderly and Petzold, 1952) is derived from the observation that hydrocarbons added to serum travel preferentially with certain of the serum components when these are separated by electrophoresis (Table VI). This effect is probably due to adsorption and

TABLE VI
Association of Two Carcinogenic Hydrocarbons with Different Serum Proteins (Wunderley *et al.*, 1952)

Serum Protein	Per Cent of Total Serum	mg. of hydrocarbon associated per g. of protein	
		20-Methylcholanthrene	3,4-Benzpyrene
Albumin	64	4.3	3.1
α_1-globulin	4.8	7.1	5.0
α_2-globulin	7.0	6.8	4.3
β-globulin	11.1	10.0	7.8
γ-globulin	13.0	0.9	1.0

not chemical combination. The fact that certain enzymes can be inhibited *in vivo* by hydrocarbons (Boyland, 1933; Rondoni and Barbieri, 1950) also indicates that at least some interaction with proteins must take

place. Creech and Franks (1937) prepared a covalent complex between proteins and 1,2,5,6-dibenzanthracene by converting the latter into the isocyanate which reacts with free amino groups in proteins; the product formed was not carcinogenic.

An analogy between the combination of the carcinogenic hydrocarbons and the absorption of dyes by cotton, which proceeds by van der Waals forces, is tempting (Bradley, 1936). However, since the structural factors governing dye adsorption are only vaguely understood, the comparison does not materially help our understanding of the mode of action of these carcinogens. High affinity by dyes seems to require a coplanar molecule (cf. Vickerstaff, 1950), and this is also one of the physical properties common to all the carcinogenic hydrocarbons. Another factor in dye absorption which has been established with some certainty is that the affinity increases with molecular size (Steinhardt, Fugitt, and Harris, 1940; Fowler *et al.*, 1952). In this respect also there exists a certain parallelism with the carcinogenic hydrocarbons, but for these there is a limit of complexity above which activity decreases (cf. Haddow, 1953). The affinity of certain dyes is enhanced on the introduction of nonpolar groups such as long alkyl chains (Alexander and Charman, 1950) and a related effect has been noted with carcinogenic hydrocarbons which are often inactivated on the introduction of polar groups. Druckrey *et al.* (1952b) stress the importance of adsorption, which they suggest occurs on a negatively charged site (i.e., the carcinogen must have a basic or positive group). They believe that the reactive center of the hydrocarbons, the so-called K region where there is a high electron density, can be considered as a basic group although the reason for this is not obvious to the writer. In support of this view they quote the observation by Windaus and Rennhak (1937) that the introduction of a negatively charged group (i.e., sulfonic acid residue) into benzpyrene results in a loss of activity. They disregard the fact reported in the same paper that the introduction of a basic group (i.e., NH_2) also deactivates this hydrocarbon. In agreement with the views of Druckrey *et al.* (1952b) it would appear, however, that in general the introduction of basic groups such as NH_2 and CN does not result in loss of activity, whereas the introduction of acidic groups, such as OH, NO_2, and COOH, brings about inactivation (cf. Badger, 1948). However, there are many exceptions to this rule, and it is not certain if any generalization is justified.

Further evidence that adsorption plays a part in the action of these carcinogens comes from a study of their metabolic products. It is found that no substitution occurs *in vivo* at their most reactive center, the K region, where chemical reaction is most probable. Boyland (cf. 1950) suggests that the hydrocarbons are combined (either by primary or secondary

valencies) to tissue components at this point and that reaction is thereby prevented.

The solubilization of aromatic hydrocarbons by purines, pyrimidines, and nucleotides (Weil-Malherbe, 1946) indicates that interaction occurs in solution. Many nonpolar materials have an increased solubility in soap solutions because they dissolve in the nonpolar parts of the soap micelles. However, this mechanism cannot apply to the solubilization by purines, etc., which do not form micelles, and the effect here is probably due to the combination of these substances with the hydrocarbons to form a soluble complex. Polyvinyl acetate becomes soluble in solutions containing alkyl sulfonates by this mechanism (Sata and Shuji, 1952). Boyland (1952a) extended these investigations to nucleic acids which were also found to solubilize aromatic hydrocarbons, although to a smaller extent. A loose combination between carcinogenic hydrocarbons and nucleic acids in aqueous solutions is therefore indicated, and according to Boyland (1952b) this absorption may be sufficient to bring about chromosome abnormalities.

2. Photodynamic Activity

In 1900 Raab, studying the toxicity of dyes for paramecia, found that the time required to kill depended on the light intensity. Following this discovery a similar action of dyes and related substances (referred to as

TABLE VII

Comparison of Carcinogenic Activity with Photodynamic Effect against Paramecia of Polycyclic Hydrocarbons
(Mottram and Donniach, 1938)

Compound Used	Carcinogenicity	Photodynamic Effect
Cholanthrene	+++	+++
3,4-Benzpyrene	+++	+++
20-Methylcholanthrene	+++	+++
2-Me-3,4-benzphenanthrene	++	+
1,2,5,6-Dibenzanthracene	++	++
1,2,5,6-Dibenzacridine	+	−
3,4-Benzphenanthrene	+	++
1,2-Benzanthracene	+	+++
Anthracene	−	+
Phenanthrene	−	−
1,9-Dimethylphenanthrene	−	−
Fluorene	−	−
1,2-Benzfluorene	−	−
Perylene	−	−
Cholesterol	−	−
Ergosterol	−	−

sensitizers) was found in many biological effects (e.g., hemolysis) and *in vitro* oxidations, all these reactions are called "photodynamic." This whole field is summarized excellently in a monograph by Blum (1941). The main criteria for photodynamic action are the need for visible light (not ultraviolet) and oxygen. The chemical reaction which occurs is a photosensitized oxidation with dissolved oxygen. Mottram and Doniach (1937a, 1938; Doniach, 1939) found that the carcinogenic hydrocarbons are 10^3 to 10^4 times as active for killing paramecia as the dyes which are normally used as photodynamic sensitizers. Hollaender *et al.* (1939) found that methylcholanthrene kills yeast cells in the presence of light, whereas

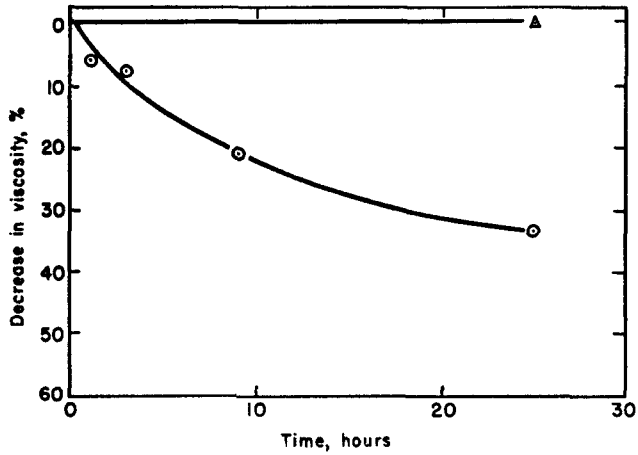


FIG. 14. Photodynamic degradation of deoxyribose nucleic acid sensitized by 1,2-benzanthracene (Koffler and Markert, 1951). *Top curve:* nucleic acid only, nucleic acid + white light; nucleic acid + 1,2 benzanthracene in the dark; all fall on the same curve. *Bottom curve:* nucleic acid + 1,2-benzanthracene in white light.

it stimulates their growth in the dark. An impressive correlation between photodynamic activity against paramecia and carcinogenicity is shown in Table VII.

In vitro, oxidation of such compounds as leuco dyes can be brought about photodynamically as can the *in vitro* inactivation of enzymes, toxins, antitoxins, and viruses (see Blum, 1941). This indicates that photodynamic action can bring about reaction with proteins and nucleic acids. In all these experiments dyes were used as sensitizers, but Rideal (1939), using a surface film technique, found that the carcinogenic hydrocarbons in the presence of light react with proteins. Recently Koffler and Markert (1951) showed that DNA is degraded photodynamically by methylcholanthrene and 1,2-benzanthracene (see Fig. 14). Polymethacrylic acid in aqueous solution is also broken down by polycyclic hydro-

carbons and as in all the other systems visible light and oxygen were necessary (Alexander and Fox, unpublished).

Photodynamic action can therefore degrade macromolecules in the same way as ionizing radiation and the carcinogenic hydrocarbons are outstandingly powerful sensitizers for these reactions. The mechanism of photodynamic action has not been fully elucidated, but Kautsky *et al.* (1933) put forward convincing evidence that the sensitizer and substrate need not come into contact and that the effect is due to activated oxygen. According to Blum (1941) the following reaction scheme, where D is the sensitizer and X the substrate (e.g., macromolecule) reacting, is in accord with these facts.

$$D + h\nu \rightarrow D' \quad \text{(activation of sensitizer)}$$
$$D' + O_2 \rightarrow D + O_2' \quad \text{(activation of oxygen)}$$
$$O_2' + X \rightarrow X \text{ oxidizes} \quad \text{(photodynamic reaction)}$$

In many ways the concept of activated oxygen is reminiscent of the earlier ionizing radiation theories that required activated water as the reacting species. When the existence of free radicals in solution was recognized, activated water was seen to be free radicals (see p. 43). It is tempting therefore to postulate free radicals as the active agent for the photodynamic process. Support for this view is found in the observations that typical radiation protective agents such as sodium thiosulfate and allylthiourea respectively inhibit the photodynamic hemolysis by dyes (Blum, 1937) and the degradation of polymethacrylic acid by 3,4-benzpyrene (Alexander and Fox, unpublished).

In the light of modern knowledge concerning the action of light a satisfactory mechanism for the production of free radicals can be proposed. The polycyclic hydrocarbons and the other photosensitizers go over on the absorption of low-energy light (i.e., visible light) into a triplet state in which the two most weakly bound electrons of the π system are unpaired. Such triplet states, which are in chemical terms biradicals, have long lifetimes (*ca.* 10^{-2} seconds) during which period they are susceptible to chemical attack. Any oxidation process must first involve such an unpaired state and accordingly can occur more readily when the unpairing has already been accomplished. An HO_2 radical could according to Bowen (1950) be formed by electron transfer in the reaction of oxygen with a molecule at the triplet level in this way:

$$D + h \rightarrow D' \quad \text{(triplet state)}$$
$$D' + O_2 \rightarrow D^+ + O_2^-$$
$$O_2^- + H_2O \rightarrow HO_2 + OH^-$$

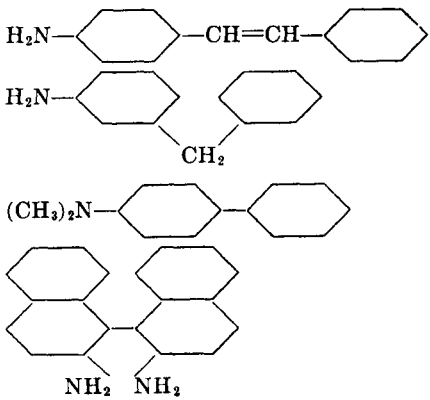
There are, however, many other reactions by which free radicals can be produced from excited dye molecules and oxygen.

The evidence that light enhances the carcinogenic action of hydrocarbons is conflicting. Maisin and de Jonghe (1934) report a greater tumor incidence on painting with 3,4-benzpyrene for rats kept in a light rather than in a dark room. However, other workers have failed to repeat this experiment (see Blum, 1941, p. 266). Mottram and Doniach (1937a) found that carcinogenic hydrocarbons produced dermatitis rapidly in the light and not in the dark. For the carcinogenic action of the hydrocarbons the necessity for light cannot be a general requirement since of course tumors can be produced by injection of these substances. Dr. C. Reid has suggested to the reviewer that adsorption of a hydrocarbon on a suitable substrate may bring about electron unpairing processes, so that the molecule can undergo in the dark those reactions which normally require light.

Developing the ideas of Schmidt and the French school, Haddow (1947) proposed that the proximate carcinogenic agent is neither the hydrocarbon nor one of its metabolites, but the energy released during the transformation from one to another; and that both physical and chemical carcinogens may be considered as sources or carriers of energy in such a form as can readily interfere with normal growth of the cell. The photodynamic activity of the hydrocarbons permits the speculation that this energy carrier is a free radical formed during the reaction of oxygen with an activated form of the hydrocarbon. As has been indicated these free radicals can degrade macromolecules such as DNA and thus lead to the interference with growth.

V. Compounds Containing Amino Groups

A number of structurally related aromatic amino compounds are carcinogenic and the most prominent members are 4-aminostilbene (I) and its derivatives, 2-aminofluorene (II), 4-dimethylaminobiphenyl (III), and 2,2′-diamino-1,1′-dinaphthyl (IV). The substituted amino azo compounds will be considered separately below.



I (Haddow *et al.*, 1948)

II (Wilson *et al.*, 1941)
(activity is retained when the CH_2 group is replaced by —O— or —S— (Miller *et al.*, 1949)

III (Miller *et al.*, 1949)

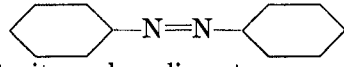
IV (Cook *et al.*, 1940)

The structural requirements for carcinogenicity have been studied in great detail for the aminostilbenes by Haddow *et al.* (1948); two criteria for activity became apparent. Firstly, the molecule must be coplanar and secondly, the 4′ position must not be substituted. The first requirement which also holds for the carcinogenic hydrocarbon indicates that adsorption on macromolecules may play a part in their action since coplanarity is known to be essential in dyes if these are to have a high affinity for fibers (see p. 58). A noteworthy feature is that all the carcinogenic amines shown contain structures which, when present in dyestuffs, lead to a high affinity (Vickerstaff, 1950). The presence of the amino group favors adsorption on acid sites, but since all the compounds are only feebly basic (i.e., pK less than 7) combination by electrostatic bonds can only be a contributory factor.

The aminostilbenes are very light-sensitive and in the presence of daylight and oxygen degrade polymethacrylic acid (Alexander and Fox, unpublished). This isolated observation suggests the possibility that photodynamic action may link them with the hydrocarbons. Although the other carcinogenic amines do not appear to have been examined as sensitizers, compounds with related structures (see Blum, 1941) do bring about photodynamic action.

Although stilbamidine has not been found to be carcinogenic, it possesses the closely related property of growth inhibition, and its consideration here may be relevant. Kopac (1947) found that stilbamidine interfered with nucleoproteins by adsorption on the DNA, thereby replacing the protein (compare with action of cytotoxic alkylating agents p. 33). Also, the antibacterial action of diamidines is thought to result from interference with nucleic acids since these powerfully and specifically antagonize their action (Bichowski-Slomnitzki, 1948). The carcinogenic amines can probably not bring about these reactions since their amino groups are much less basic than the guanidino groups of stilbamidine.

1. Combination of Amino Azo Compounds with Body Proteins

The dye butter yellow,  , and many compounds related to it produce liver tumors when fed and the specificity of the site of action suggests that the actual carcinogen is a metabolic product or that one of the stages of carcinogenesis required a metabolic reaction confined to the liver. This whole field has been reviewed by E. C. and J. A. Miller in Vol. 1 of this series of reports. These compounds have not been shown to undergo any reaction *in vitro* with macromolecules although again they have a coplanar structure which favors adsorption and the azo benzene configuration figures in many dyestuffs. *In vivo*, however, Miller and Miller (summarized 1952, 1953)

were able to demonstrate that these compounds chemically combined with liver proteins, and a significant correlation between this reaction and their carcinogenicity was found. Thus, combination only occurs with proteins of the susceptible organ, the liver; no combination is found in the livers of animals such as rabbits and certain strains of rats which are not susceptible to these dyes; finally in the tumor mass no protein bound dye could be found though uncombined dye was present.

Miller and Miller consider that a major metabolite is the highly reactive N-hydroxymethyl derivative. Although such compounds are unstable and decompose rapidly to the amine and formaldehyde, these workers consider that some of this derivative at least may combine with proteins via a reactive hydrogen of the amino, guanidine, imidazol, or phenolic hydroxyl groups. Reaction with latter groups (i.e., the side chains from tyrosine) seems the most probable since the $>N—CH_2—N<$ link formed between dye and amino groups is known to be very unstable (Fraenkel-Conrat *et al.*, 1948), and would probably not survive extraction.

A reaction of this type has been demonstrated in wool. In the presence of formaldehyde the ϵ-amino groups are converted to N-methylhydroxy groups which then react with the phenolic hydroxy group of tyrosine to give a new crosslink, the existence of which was established by direct analysis (Alexander *et al.*, 1951b). Also, by treating wool in the presence of formaldehyde with a number of different aromatic amines these were chemically combined with the protein via the phenolic hydroxyl groups of tyrosine (Johnson, 1952).

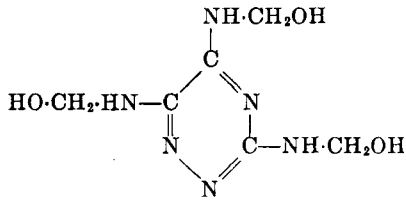
In view of the high reactivity of N-hydroxymethyl derivatives the failure of the liver of resistant animals and of tumor tissue to combine with the azo dyes is unlikely to be due to the absence of reactive groups in these proteins. Absence of the enzyme system responsible for converting the dye into the reactive form seems a more plausible explanation.

In the view of Miller and Miller (1952) the discovery of *in vivo* combination of the amino-azo dyes and of the carcinogenic hydrocarbons with proteins (see p. 56) supports the theory that carcinogenesis results from protein (i.e., enzyme) deletion which fails to kill the cell but deprives it of a growth-controlling factor. Since an *in vivo* reaction with nucleic acids has only been found with ionizing radiations (Limperos and Mosher, 1950), the view that specific proteins have to be inactivated appears to be more firmly founded than the suggestion arising from the chromosome hypothesis that reaction or destruction of nucleic acids is necessary. However, the possibility that the reactions leading to carcinogenesis need only occur in one or two cells may render deductions from observed gross changes unsound.

VI. Carcinogenic Polymers

In a search for cytotoxic agents Hendry *et al.* (1951a) studied compounds derived from melamine (and substituted melamines) and formaldehyde. A mixture of different substances is obtained in these reactions, and the actual materials tested were ill defined.

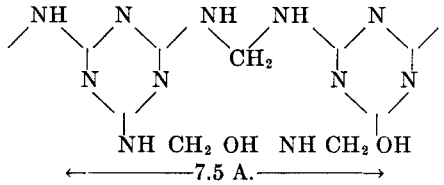
An active substance was found when the ratio of melamine to formaldehyde used was 1,3, and although a number of different compounds must be formed simultaneously Hendry *et al.* consider that they were in fact dealing largely with trimethylolmelamine



The compound used was stated to be water insoluble and was applied in the biological experiments as a suspension. A pure specimen of trimethylolmelamine is water soluble, and a 4.75% solution can be obtained at room temperature (Dudley and Lynn, 1946). This substance is, however, very unstable and readily polymerizes, and the method of preparation used by Hendry *et al.* would favor polymerization. It is probable therefore that these workers used a polymer. Since maximum activity is found with melamines containing an average of three methylol groups, it is possible that two of these take part in the polymerization and the third is available for reaction. N-methylol groups are highly reactive and have been shown to react with proteins (see p. 64) so that the polymeric derivative of trimethylolmelamine can combine with macromolecules. Unfortunately, nothing is known concerning the structure of these polymers or their molecular weight. In general melamine formaldehyde complexes polymerize in two not very distinct stages. At first a lightly cross-linked substance is obtained which then slowly polymerizes further with the evolution of formaldehyde. The possibility that the cytotoxic activity of substances tested was the result of a continuous slow evolution of formaldehyde cannot be ruled out.

The mode of action proposed by Hendry *et al.* is similar to that put forward for the action of the cytotoxic alkylating agents, namely that the trimethylolmelamine polymerizes *in situ* to give a polymer with reactive side chains which then combine with vital macromolecules. By postulating a suitable polymerization mechanism a hypothetical structure with

reactive side chains repeating at 7.5 A. was postulated as being formed *in situ*.



There is, however, no evidence that a reaction of this type occurs, especially if starting material was already partly polymerized. Other aspects of this theory have been discussed on p. 20.

Implantation of Bakelite disks for long periods produced fibrosarcoma (Turner, 1941), and this unexpected finding was extended by Oppenheimer *et al.* (1948), who observed that sarcomas appeared at or near the site where cellophane was embedded. In a systematic investigation these workers (1952, 1953) enlarged their findings and record tumor production on implantation of films of both commercial and highly purified cellophane, polyethylene, polytetrafluoroethylene (Teflon), polystyrene, nylon, polyglycol terephthalate (Dacron), a silicon polymer (Silastic), and polyvinyl chloride. Druckrey *et al.* (1952b) confirmed the production of tumors with cellophane and obtained similar results with Perlon (a polyamide resembling nylon). In view of the chemical inertness and diversity of constitution of the films used, it is difficult to see a chemical mechanism. The suggestion (Druckrey *et al.*, 1952b) that these polymers hydrogen bond to macromolecules and that they function by the same mechanism which Hendry *et al.* (see p. 20) postulate for the cytotoxic alkylating agents cannot be sustained. Although nylon and cellophane can conceivably combine in this way, none of the other polymers listed above as producing tumors have groupings capable of forming hydrogen bonds. Since these substances do not produce tumors when embedded as fibers (e.g., cotton linters or surgical cotton) and since preliminary experiments (Oppenheimer *et al.*, 1953) indicate that the polymers in the form of perforated films or woven textiles are much less active, a physical mechanism initiated by flexible films seems to be indicated. Although mechanical irritation is unlikely to be the cause, it may be that the films prevent free interchange of metabolites and metabolic products with the rest of the body at the site of implantation, and this may conceivably interfere with normal development of the cell. If this should prove to be the case, it may have important implications for the spontaneous causation of cancer.

REFERENCES

Abel, E. 1948. *Monatsh.* **79,** 457–468.

Adams, D. H., and Thompson, R. H. S. 1948. *Biochem. J.* **42,** 170–175.

Alexander, P. 1950. *Brit. Empire Cancer Campaign Rept.* **28,** 64.

Alexander, P. 1952. *Nature* **169,** 226–228.

Alexander, P. 1953. *Biochim. et Biophys. Acta* **10,** 595–599.

Alexander, P., Carter, D., Earland, C., and Ford, O. E. 1951a. *Biochem. J.* **48,** 629–637.

Alexander, P., Carter, D., and Johnson, K. G. 1951b. *Biochem. J.* **48,** 435–441.

Alexander, P., and Charman, D. C. 1950. *Textile Research J.* **11,** 761–770.

Alexander, P., and Fox, M. 1952a. *Nature* **169,** 572–574.

Alexander, P., and Fox, M. 1952b. *Nature* **170,** 1022–1023.

Alexander, P., and Fox, M. 1953a. (In course of publication).

Alexander, P., and Fox, M. 1953b. *J. chim. phys.* **50,** 415–422.

Alexander, P., Fox, M., Smith, F. L., and Stacey, K. A. 1952. *Biochem. J.* **52,** 177–184.

Alexander, P., and Hitch, S. F. 1952. *Biochim. et Biophys. Acta* **9,** 229–236.

Alexander, P., and Ross, W. C. J. 1951. *Brit. Empire Cancer Campaign Repts.* **29,** 11.

Alexander, P., and Whewell, C. S. 1947. *British Intelligence Objectives Sub-committee Report,* No. 1472. H. M. Stationery Office, London.

Alper, T. 1953. *Trans. Faraday Soc.* **49,** 328–329.

Anderson, R. S. 1953. *Trans. Faraday Soc.* **49,** 333.

Arnow, L. E. 1935. *J. Biol. Chem.* **110,** 43.

Arnow, L. E. 1936. *Physiol. Revs.* **16,** 671–685.

Astbury, W. T., and Woods, H. J. 1943. *Phil. Trans. Roy. Soc.* **232,** 333–394.

Avery, O. T., MacLeod, C. M., and McCarty, M. 1944. *J. Exptl. Med.* **79,** 137–158.

Bacon, R. G. R. 1946. *Trans. Faraday Soc.* **42,** 140–155.

Badger, G. M. 1948. *Brit. J. of Cancer* **2,** 309–350.

Banks, T. E., Boursnell, J. C., Francis, G. E., Hopwood, F. L., and Wormall, A. 1946. *Biochem. J.* **40,** 745–756.

Barron, E. S. G. 1952. *Symposium on Radiobiology.* John Wiley & Sons, N.Y., pp. 216–240.

Berenblum, I., and Wormall, A. 1939. *Biochem. J.* **33,** 75.

Benoit, H. 1950. *J. chim. phys.* **47,** 720.

Benoit, H. 1951. *Ann. de Phys.* **6,** 591.

Bestian, W. 1950. *Ann.* **566,** 210–229.

Bichowski-Stomnitzki, L. 1948. *J. Bact.* **55,** 33–41.

Biesele, J. J., Philips, F. S., Thiersch, J. B., Burchenal, J. H., Buckley, S. M., and Stock, C. C. 1950. *Nature* **166,** 1112–1113.

Bjorksten, J. 1951. *Advances in Protein Chem.* **6,** 343–384.

Blackburn, S., Carter, S. E., and Phillips, H. 1941. *Biochem. J.* **35,** 627.

Blum, H. F. 1941. *Photodynamic Action and Diseases Caused by Light.* Reinhold Publishing Corp., N.Y.

Boivin, A. 1947. *Cold Spring Harbor Symposia Quant. Biol.* **12,** 7–17.

Bonème, R., and Magat, M. 1951. *Compt. rend.* **232,** 1104–1106.

Boursnell, J. C. 1948. *Biochem. Soc. Symposia* **2,** 8–15.

Boursnell, J. C., Francis, G. E., and Wormall, A. 1946. *Biochem. J.* **40,** 765–768.

Bowen, E. J. 1950. *Photochem. in Relation to Textiles.* Soc. Dyers Colourists, Bradford, pp. 29–33.

Boyland, E. 1933. *Biochem. J.* **17,** 791–801.

Boyland, E. 1948. *Biochem. Soc. Symposia* **2,** 61–70.

Boyland, E. 1950. *Biochem. Soc. Symposia* **5**, 40–53.
Boyland, E. 1952a. *Cancer Research* **12**, 77–84.
Boyland, E. 1952b. *Endeavour* **11**, No. 42.
Boyland, E., Goss, G. C. L., and Williams-Ashmann, H. G. 1951. *Biochem. J.* **49**, 321–324.
Bradley, W. 1936. *Nature* **137**, 404–405.
Brown, D. M., and Todd, A. R. 1952. *J. Chem. Soc.* 52–58.
Bullock, K. 1952. (Private communication.)
Burton, M. 1952. *Symposium on Radiobiology.* John Wiley & Sons, N.Y., pp. 117–139.
Butler, J. A. V. 1950. *Nature* **166**, 18–19.
Butler, J. A. V. 1951. *Brit. Empire Cancer Campaign Repts.* **28**, 63.
Butler, J. A. V., and Conway, B. E. 1950. *J. Chem. Soc.* 3418–3421.
Butler, J. A. V., Conway, B. E., and James, D. W. F. 1951. *J. chim. phys.* **48**, 609–612.
Butler, J. A. V., Gilbert, L. A., and James, D. W. F. 1952. *J. Chem. Soc.* 3268–3273.
Butler, J. A. V., Gilbert, L. A., and Smith, K. A. 1950. *Nature* **165**, 714–715.
Butler, J. A. V., and James, D. W. F. 1951. *Nature* **167**, 844–845.
Butler, J. A. V., and Press, E. M. 1952. *J. Chem. Soc.* 626–631.
Butler, J. A. V., and Smith, K. A. 1950. *J. Chem. Soc.* 3411–3418.
Capp, C. W., and Speakman, J. B. 1949. *J. Soc. Dyers Colourists* **65**, 402–411.
Carlene, P. W., Fern, A. S., and Vickerstaff, T. 1947. *J. Soc. Dyers Colourists* **63**, 388–393.
Carpenter, F. H., Wood, J. L., Stevens, C. M., and du Vigenaud, V. 1948. *J. Am. Chem. Soc.* **70**, 2551–2553.
Chanutin, A., and Gjessing, E. C. 1946. *Cancer Research* **6**, 599–601.
Charlesby, A. 1952. *Proc. Roy. Soc. (London)* **215**, 187–214.
Conway, B. E., and Butler, J. A. V. 1952. *J. Chem. Soc.* 834–838.
Conway, B. E., and Butler, J. A. V. 1953. *Trans. Faraday Soc.* **49**, 327–328.
Conway, B. E., Gilbert, L., and Butler, J. A. V. 1950. *J. Chem. Soc.* 3421–3425.
Cook, J. W., Hewett, C. L., and Kennaway, N. M. 1940. *Am. J. Cancer* **40**, 62–77.
Corey, R. B. 1938. *J. Am. Chem. Soc.* **60**, 1598–1604.
Crabtree, H. G. 1944. *Cancer Research* **4**, 688.
Crabtree, H. G. 1945. *Cancer Research* **5**, 346.
Crabtree, H. G. 1948. *Brit. J. Cancer* **2**, 287.
Creech, H. J., and Franks, W. R. 1937. *Am. J. Cancer* **30**, 555.
Cullumbine, H., and Rydon, H. N. 1946. *Brit. J. Exptl. Path.* **27**, 33–46.
Daimler, B. H. 1952. *Kolloid Z.* **127**, 97.
Dainton, F. S. 1952. *J. Chem. Soc.* 1533–1546.
Dainton, F. S., and Holmes, B. 1950. *Nature* **165**, 266–269.
Dale, W. M. 1952. *Trans. Faraday Soc. Symposia* **12**, 293–299.
Dale, W. M., and Davies, J. V. 1949. *Nature* **163**, 64; 1951. *Biochem. J.* **48**, 129–132.
Davies, S. B., and Ross, W. F. 1947. *J. Am. Chem. Soc.* **69**, 1177–1187.
Davis, W., and Ross, W. C. J. 1952. *J. Chem. Soc.* 4296–4300.
Davis, W., and Ross, W. C. J. 1950. *J. Chem. Soc.* 3056–3061.
Deuel, H. 1947. *Helv. Chim. Acta* **30**, 1523–1543.
Deuel, H., and Neukom, H. 1949. *J. Polymer Sci.* **4**, 755–757.
Devik, F. 1952. *Brit. J. Radiology* **25**, 481–484.
Doniach, O. 1939. *Brit. J. Exptl. Path.* **20**, 227–235.
Druckrey, H., Schmähl, D., and Danneberg, P. 1952a. *Z. Naturfrsch.* **7b**, 353–356.
Druckrey, H., Schmähl, D., and Danneberg, P. 1952b. *Naturwissenschaften* **39**, 393–397.

Dudley, J. R., and Lynn, J. E. 1946. *Fibrous Prot. Symp.* Soc. Dyers Colourists, Bradford, pp. 215–220.
Dustin, P. 1947. *Nature* **159,** 794–796.
Elmore, D. T., Gulland, J. M., Jordan, D. O., and Taylor, H. F. W. 1948. *Biochem. J.* **42,** 308–316.
Ephrussi-Taylor, H. 1951. *Cold Spring Harbor Symposia Quant. Biol.* **16,** 445–456.
Errera, M. 1947. *Cold Spring Harbor Symposia Quant. Biol.* **12,** 60–63.
Everett, J. L., and Kon, G. A. R. 1950. *J. Chem. Soc.* 3131–3135.
Everett, J. L., Roberts, J. J., and Ross, W. C. J. 1953. *J. Chem. Soc.* 2386–2392.
Faraday Society Discussion. 1952. No. 12.
Fearnley, C., and Speakman, J. B. 1950. *Nature* **166,** 743.
Felix, K., Fisher, H., Krekels, A., and Mohr. R. 1951. *Z. physiol. Chemie* **287,** 224–234; **289,** 10–19.
Felix, K., Hartleib, J., and Krekels, A. 1952. *Z. physiol. chemie* **290,** 66–69.
Fieser, L. F. 1941. Univ. of Pennsylvania, Bicentenary Lecture.
Fowler, J. A., Michie, A. G. H., Vickerstaff, T. 1951. *Melliand Textilber.* **32,** 293–302.
Fraenkel-Conrat, H. 1944. *J. Biol. Chem.* **154,** 227–238.
Fraenkel-Conrat, H., and Olcott, H. S. 1946. *J. Biol. Chem.* **161,** 259–268.
Fraenkel-Conrat, H., and Olcott, H. S. 1948. *J. Biol. Chem.* **174,** 827–843.
Fricke, H. 1938. *Cold Spring Harbor Symposia Quant. Biol.* **6,** 164–169.
Fruton, J. S., Stein, W. H., and Bergmann, M. 1946. *J. Org. Chem.* **11,** 559–570
Gjessing, E. C., and Chanutin, A. 1946. *Cancer Research* **6,** 593–598.
Goldacre, R. J., Loveless, A., and Ross, W. C. J. 1949. *Nature* **163,** 667–669.
Gray, L. H. 1952. *Brit. J. Radiology* **25,** 235–244.
Greenstein, J. P., and Jenrette, W. V. 1941. *Cold Spring Harbor Symposia Quant. Biol.* **9,** 236–252.
Haber, F., and Weiss, J. 1934. *Proc. Roy. Soc.* (*London*) **A147,** 332.
Haddow, A. 1935. *Nature* **136,** 868–869.
Haddow, A. 1947. *Growth Symposium* **11,** 339–358.
Haddow, A. 1949. *Proc. 1st Natl. Cancer Conf.* 88–94.
Haddow, A. 1951. *Proc. Roy. Soc. Med.* **44,** 263.
Haddow, A. 1953. Physiopathology of Cancer. P. Hoeber, N. Y.
Haddow, A., Everett, J. L., and Ross, W. C. J. 1951. *Brit. Empire Cancer Campaign Repts.* **29,** 5.
Haddow, A., Harris, R. J. C., Kon, G. A. R., and Roe, E. M. F. 1948. *Phil. Trans. Roy Soc.* **241,** 147–195.
Haddow, A., Kon, G. A. R., and Ross, W. C. J. 1948. *Nature* **162,** 824–825.
Haddow, A., and Timmis, G. M. 1950. *Brit. Empire Cancer Campaign Repts.* **28,** 59.
Haddow, A., and Timmis, G. M. 1951. *Acta Unio Intern. contra Cancrum VII* **3,** 469–471.
Haddow, A., and Timmis, G. M. 1953. *Lancet.* **1,** 207.
Hanby, W. E., and Rydon, H. N. 1947. *J. Chem. Soc.* 513–519.
Hartwell, J. L. 1945. *J. Natl. Cancer Inst.* **6,** 319–324.
Hartwell, J. L. 1951. *Survey of Compounds Which Have Been Tested for Carcinogenic Activity.* U.S. Public Health Service publication, No. 149.
Haurowitz, F. 1950. The Chemistry and Biology of Proteins. Academic Press, N.Y.
Heidelberger, C., and Jones, H. B. 1948. *Cancer* **1,** 252–260.
Heidelberger, C., and Weiss, S. M. 1951. *Cancer Research* **11,** 885–891.
Hendry, J. A., Rose, F. L., and Walpole, A. L. 1950. *Nature* **165,** 993–996.
Hendry, J. A., Rose, F. L., and Walpole, A. L. 1951a. *Brit. J. Pharmacol.* **6,** 201–234.

Hendry, I. A., Rose, F. L., and Walpole, A. L. 1951b. *Brit. J. Pharmacol.* **6**, 235–255.
Hendry, J. A., Rose, F. L., and Walpole, A. L. 1951c. *Brit. J. Pharmacol.* **6**, 357–410.
Herriott, R. M. 1948. *J. Gen. Physiol.* **32**, 221–239.
Hollaender, A., Cole, P. A., and Bracket, F. S. 1939. *Am. J. Cancer* **37**, 265.
Hudson, R. F., Marshall, A., and Timmis, G. M. 1953. (Private communication.)
Jensen, E. V. 1950. *Trans. 5th Conf. Biol. Autoxidants.* Josiah Macy Jr. Foundation, N.Y., pp. 159–186.
Johnson, K. G. 1952. Ph.D. Thesis Univ. of London.
Kahler, H. 1948. *J. Phys. Chem.* **52**, 676–689.
Kautsky, H., de Bruyn, H., Nauwirth, R., and Baumeister, W. 1933. *Ber.* **66**, 1588–1600.
Kenokh, M. A. 1950. *Zhur. Obshchei Khim.* **20**, 1560–1567.
Koenig, V. L., and Perrin, J. D. 1952. *Arch. Biochem.* **38**, 105–119.
Koffler, H., and Markert, I. L. 1951. *Proc. Soc. Exptl. Biol. Med.* **76**, 90–92.
Koller, P. C., and Casarini, A. 1952. *Brit. J. Cancer* **6**, 173–185.
Kon, G. A. R., and Roberts, J. J. 1950. *J. Chem. Soc.* 978–982.
Kopac, M. J. 1947. *Approaches to Tumor Chemotherapy.* Am. Assoc. Advancement Sci., Washington, D.C., pp. 27–57.
Kuhn, W. 1932. *Z. physik. Chem.* **161**, 1–32.
Kuhn, W., and Kuhn, H. 1945. *Helv. Chim. Acta* **28**, 97–127; 1533–1579.
Kuhn, W., and Kuhn, H. 1948. *J. Colloid Sci.* **3**, 11–32.
Kuhn, W., Künzle, O., and Katchalsky, A., 1948. *Helv. Chim. Acta* **31**, 1994–2037.
Lea, D. M. 1946. *Actions of Radiations on Living Cells.* Cambridge Univ. Press, Cambridge.
Limperos, G., and Mosher, W. A. 1950. *Am. J. Roentgenol. Radium Therap.* **63**, 691–700.
Linderstrom-Lang, K. 1933. *Ergeb. Physiol. exptl. Pharmakol.* **35**, 415.
Little, K. 1952. *Nature* **170**, 1075–1076.
Loveless, A. 1951. *Nature* **167**, 338–342.
MacDonald, M. R. 1953. *Trans. Faraday Soc.* (In press.)
Maisin, J., and de Jonghe, A. 1934. *Compt. rend. soc. biol.*, **117**, 111.
Meyer, K. H. 1951. *Experientia* **7**, 361–371.
Meyer, K. H. 1951. *Z. Elektrochem.* **55**, 453–459.
Miller, E. C. 1951. *Cancer Research* **11**, 100–108.
Miller, E. C., Miller, J. A., Saudin, R. B., and Brown, R. K. 1949. *Cancer Research* **9**, 504–509.
Miller, E. C., and Miller, J. A. 1952. *Cancer Research* **12**, 547–556.
Miller, J. A., and Miller, E. C. 1953. *Advances in Cancer Research* **1**, 339–396.
Mosher, W. A. 1950. *Trans. 5th Conf. Biol. Autoxidants.* Josiah Macy Jr. Foundation, N.Y., p. 74.
Mottram, J. C., and Doniach, I. 1937a. *Nature* **140**, 588.
Mottram, J. C., and Doniach, I. 1937b. *Nature* **140**, 933.
Mottram, J. C., and Doniach, I. 1938. *Lancet* **234**, 1156–1158.
Needham, D. M. 1948. *Biochem. Soc. Symposia* **2**, 16–27.
Neuberger, A., Perone, J. C., and Slack, H. G. B. 1951. *Biochem. J.* **49**, 199–204.
Northrop, F. H. 1934. *J. gen. physiol.* **17**, 359–363.
Ogston, A. G. 1948. *Trans. Faraday Soc.* **44**, 45–52.
Oppenheimer, B. S., Oppenheimer, E. T., and Stout, A. D. 1948. *Proc. Soc. Exptl. Biol. Med.* **67**, 33; 1952. **79**, 366–369.
Oppenheimer, B. S., Oppenheimer, E. T., Stout, A. P., and Danishefsky, I. 1953. *Science*, **118**, 305–306.

Ormsbee, R. A., Henriques, F. C., and Ball, E. G. 1949. *Arch. Biochem.* **21,** 301–312.
Patterson, W. I., Geiger, W. B., Mizell, L. R., and Harris, M. 1940. *Bur. Standards J. Research* **25,** 451–462.
Perry, S. V., and Reed, R. 1947. *Biochim. et Biophys. Acta* **1,** 379.
Peters, R. A. 1947. *Nature* **159,** 149–151.
Peters, R. A., and Wakelin, R. W. 1947. *Biochem. J.* **41,** 550–555.
Pfeifer, H. 1936. *Physics* **7,** 302.
Philips, F. S. 1950. *J. Pharmacol. Exptl. Therap.* **99,** 281–325.
Philips, F. S., and Thiersch, J. B. 1950. *J. Pharmacol. Exptl. Therap.* **100,** 398–407.
Pirie, A. 1947. *Biochem. J.* **41,** 185–190.
Porter, K. R., and Hawn, Z. 1949. *J. Exptl. Med.* **90,** 225.
Raab, O. 1900. *Z. Biol.* **39,** 524.
Rasmussen, K. E. 1934. *Z. physiol. Chem.* **224,** 97.
Reichmann, M. E., Bunce, B. H., and Doty, P. 1953. *J. Polymer Sci.* **10,** 109–119.
Reimann, S. P., and Hall, E. M. 1936. *Arch. Path.* **22,** 55.
Rideal, E. K. 1939. *Science* **90,** 217–225.
Rollenaal, H. M., Billamy, W. D., and Baldwin, T. H. 1951. *Nature* **169,** 694–695.
Rondoni, P., and Barbieri, G. P. 1950. *Enzymologia* **14,** 10–14.
Ross, W. C. J. 1949. *J. Chem. Soc.* 2589–2596.
Ross, W. C. J. 1950. *J. Chem. Soc.* 2257–2272.
Ross, W. C. J. 1953. *Advances in Cancer Research* **1,** 397–449.
Rutherford, H. A., Patterson, W. I., and Harris, M. 1940. *Bur. Standards J. Research* **25,** 451–459.
Sata, N., and Shuji, S. 1952. *Kolloid Z.* **128,** 154–158.
Scholes, G., and Weiss, J. 1952. *Exptl. Cellular Research* **Suppl. 2,** 219–241.
Schwander, H., and Cerf, R. 1951. *Helv. Chim. Acta* **34,** 436–445.
Schwander, H., and Signer, R. 1951. *Helv. Chim. Acta* **34,** 1344–1350.
Sheraga, H. A., and Nims, L. F. 1952. *Arch. Biochem.* **36,** 336–344.
Sisman, O., and Bopp, C. D. 1951. Oak Ridge National Lab. Rept. 928 (Physics).
Smith, D. B., and Butler, G. C. 1951. *J. Am. Chem. Soc.* **73,** 258–261.
Sokolov, J. 1940. *Biokhimiya* **5,** 401–407.
Southern Research Inst. 1952. Cancer Chemotherapy, Prog. Rept. Aug. 15.
Sparrow, A. H., and Rosenfeld, F. M. 1946. *Science* **104,** 245–246.
Stedman, E., and Stedman, E. 1947. *Cold Spring Harbor Symposia Quant. Biol.* **12,** 224–236.
Stein, W. H., and Fruton, J. S. 1946. *J. Org. Chem.* **11,** 719–735.
Steinhard, J., Fugitt, C. H., and Harris, M. 1941. *J. Research Natl. Bur. Standards* **26,** 293.
Stern, K. G. 1949. *Exptl. Cellular Research* **Suppl. 1,** 97–101.
Stevens, C. M., and Mylroie, A. 1952. *Biochim. et Biophys. Acta* **8,** 325–331.
Stevens, C. M., McKennis, H., and du Vigneaud, V. 1948a. *J. Am. Chem. Soc.* **70,** 2556–2559.
Stevens, C. M., Wood, J. L., Rachele, J. R., and du Vigneaud, V. 1948b. *J. Am. Chem. Soc.* **70,** 2554–2556.
Stora, C., Brigando, J., Schwartzkopf, O., and Genin, H. 1947. *Compt. rend.* **224,** 1367–1369.
Svedberg, I., and Brohult, S. 1939. *Nature* **143,** 938–939.
Taylor, B., Greenstein, J. P., and Hollaender, A. E. 1948. *Arch. Biochem.* **16,** 19–31.
Timmis, G. M. 1948. Brit. Pat. Appl. 20464.
Timmis, G. M. 1951. *12th Intern. Congr. Pure and Applied Chem. Abst.*, pp. 334–335.
Turner, F. C. 1941. *J. Natl. Cancer Inst.* **2,** 81.

Vickerstaff, T. 1950. *The Physical Chemistry of Dyeing.* Oliver and Boyd, London.
Watkins, W. M., and Wormall, A. 1952. *Biochem. J.* **52,** 365–377.
Weil-Malherbe, H. 1946. *Biochem. J.* **40,** 351–363.
Weiss, J., Scholes, G., and Stein, G. 1949. *Nature* **164,** 709–710.
Wilson, R. H., DeEds, F., and Cox, A. J. 1941. *Cancer Research* **1,** 595–608.
Windaus, A., and Rennhak, S. 1937. *Z. physiol. Chem.* **249,** 256.
Wood, S. L., and Fiesre, L. F. 1940. *J. Am. Chem. Soc.* **62,** 2674–2681.
Wunderly, C. H., and Petzold, F. A. 1952. *Naturwissenschaften* **39,** 493–494.
Young, E. G., Campbell, R. B. 1947. *Can. J. Research* **25,** 37–48.

Chemical Constitution and Carcinogenic Activity

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CONTENTS

I. Introduction

The study of chemical carcinogenesis dates from 1915 when cancer was first produced experimentally by the long-continued application of coal tar to the ears of rabbits, but it was not until 1930 that cancer was produced by the application of a pure polycyclic aromatic hydrocarbon, 1,2,5,6-dibenzanthracene, to the skin of mice. It was soon shown that many other related compounds are also active in this respect, and several hundred substances are now known to be carcinogenic. The majority of

these are polycyclic compounds; but several other types of carcinogen have also been discovered. These include various azo compounds, aromatic amines, aminostilbenes, nitrogen mustards, urethanes, various aliphatic compounds, and a few inorganic salts.

It is a commonplace in all studies of the relation between chemical constitution and biological action that the same biological end result can frequently be brought about by different classes of chemical compound acting by different mechanisms, and it must not be supposed that the different classes of chemical carcinogen act primarily in the same way. It would be idle, therefore, to attempt to find any chemical relationship between, say, the polycyclic aromatic compounds (which produce tumors essentially at the site of application) and the azo compounds (which do not produce tumors at the site of application, but only in the liver). On the other hand, it is not unreasonable to study the relationship between chemical constitution and carcinogenic activity within each class of carcinogen, and it is to be hoped that such studies will prove of value in elucidating the mechanism of carcinogenesis.

Considerable progress has been made in the study of the relationship between chemical constitution and carcinogenic activity in recent years, and interest in this aspect of cancer research continues unabated. This progress has been made possible by the continued accumulation of biological data, by the study of the reactions and properties of the carcinogens, and by the application of quantum mechanics to the study of these substances. The present article attempts to summarize these advances. Only extrinsic factors are considered, and special attention is devoted to the polycyclic aromatic compounds, and to the azo compounds. Up to the present these two groups of chemical carcinogens have received most attention from research workers interested in this topic.

For other reviews in this field see Badger (1948), Badger and Lewis (1952), Cook (1939, 1943), Cook, Haslewood, Hewett, Hieger, Kennaway, and Mayneord (1937), Cook and Kennaway (1938, 1940), Fieser (1938), Fieser, Fieser, Hershberg, Newman, Seligman, and Shear (1937), Greenstein (1947), Haddow (1947), Haddow and Kon (1947).

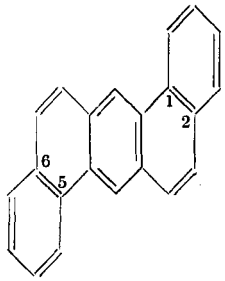
In addition, Hartwell (1951) has published a Survey of Compounds Which Have Been Tested for Carcinogenic Activity.

II. Historical

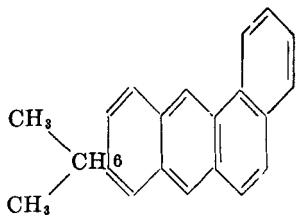
1. Polycyclic Aromatic Hydrocarbons

Soot, coal tar, shale oil, and some other complex industrial materials have long been known to be implicated in certain "industrial cancers." Bloch and Dreifuss (1921) showed that the carcinogenic factor in coal tar

is concentrated in the high-boiling fractions, and that it is free from nitrogen, arsenic, and sulfur. Kennaway (1924, 1925) was able to prepare a number of artificial carcinogenic tars, some of them containing only carbon and hydrogen. When it was found that all the carcinogenic tars show three characteristic fluorescence bands similar to but not identical with that given by 1,2-benzanthracene, it became clear that the carcinogenic factor must be a polycyclic aromatic hydrocarbon (Hieger, 1930). Several synthetic hydrocarbons, including 1,2,5,6-dibenzanthracene (I), 3′-methyl-1,2,5,6-dibenzanthracene and 6-isopropyl-1,2-benzanthracene (II), were accordingly tested by application in benzene solution to the skin of mice. After lengthy latent periods tumors were produced by all three compounds named (Cook, Hieger, Kennaway, and Mayneord, 1932; Cook, 1932).



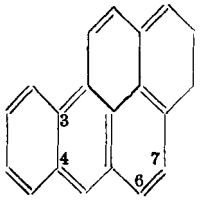
(I)



(II)

Three years later, in 1933, after a lengthy series of purification processes, a potent cancer-producing hydrocarbon was isolated from coal tar (Cook, Hewett, and Hieger, 1933). It was identified as 3,4-benzpyrene (III) and its structure was confirmed by its synthesis from pyrene. 3,4-Benzpyrene is one of the most important of all the chemical carcinogens, particularly in view of its wide distribution.* In recent years it has been identified in domestic soot (Goulden and Tipler, 1949), in processed rubber (Falk *et al.*, 1951), in carbon blacks (Falk and Steiner, 1952), and it is also known to be a constituent of the atmospheric dust in cities (Waller, 1952). Incidentally, it has been known for some years that atmospheric dust is cancer producing (Leiter and Shear, 1942).

* 3,4-Benzpyrene is not the only cancer-producing substance in coal tar. Skin tumors in rabbits are more readily produced with coal tar than with benzpyrene, and Berenblum and Schoental (1947) have shown conclusively that this is due to the presence of an additional carcinogen in the coal tar. This additional carcinogen, which also seems to be a polycyclic aromatic hydrocarbon, is much more active toward the skin of rabbits than is 3,4-benzpyrene; but the reverse applies when the skin of mice is used as test material. This additional carcinogenic factor in coal tar has not, however, been isolated in a pure condition.



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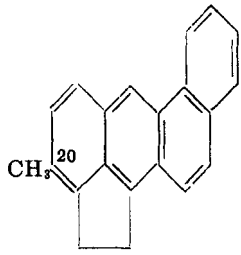


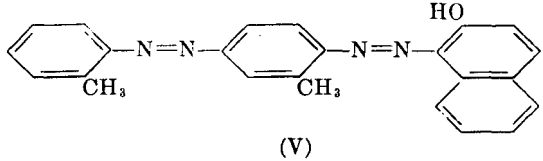
(IV)

Not long after the isolation of 3,4-benzpyrene, a potent carcinogenic hydrocarbon, 20-methylcholanthrene (IV), was produced by a series of degradations from deoxycholic acid, a normal constituent of human bile (Barry *et al.*, 1935). This raised the question whether polycyclic carcinogens might be produced *in vivo* by some abnormal metabolic process, and the result was that the study of chemical carcinogenesis was further stimulated.

Hundreds of related polycyclic aromatic compounds have now been synthesized and tested for carcinogenic activity, and many of these will be mentioned in succeeding pages.

2. Azo Compounds

The discovery of the carcinogenic azo compounds originated from an early observation (Fischer, 1906) that the azo dye Scarlet Red (V) produces a cellular proliferation, but not malignancy, in the ears of rabbits. It was subsequently found that the active part of the Scarlet Red molecule is *o*-aminoazotoluene (VI) and carcinogenic activity was first demonstrated in 1931 by the production of hepatomas in rats following the administration of this compound with the food (Yoshida, 1933, 1934).



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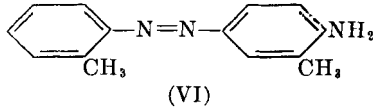
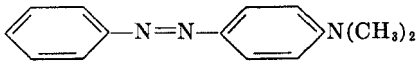


(VI)

Other derivatives of azobenzene were examined and it was soon reported that 4-dimethylaminoazobenzene (VII, "butter yellow") is even more effective than *o*-aminoazotoluene in producing liver tumors in rats (Kinosita, 1937). Many scores of related azo compounds have now

been synthesized and tested and many of these have been shown to be potent liver carcinogens.



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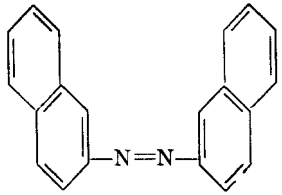
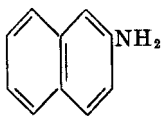


(VIII)

Nearly all the active compounds of this class contain an amino or alkylamino group; but such a group does not appear to be essential for activity of this type for 2,2′-azonaphthalene (VIII) has also been shown to produce liver tumors in a high percentage of the mice treated (Cook, Hewett, Kennaway, and Kennaway, 1940). This compound was originally tested as it was thought that it might be present as an impurity in commercial 2-naphthylamine and might therefore be implicated in the cancers of the bladder to which operatives in the dyestuffs industry are particularly liable. No bladder tumors were obtained with this compound in experimental animals, however.

3. Amino Compounds

The recognition that cancer of the bladder is an industrial hazard in the aniline dye industry led to the discovery of another class of chemical carcinogens, the amino compounds. Although several compounds may be implicated in the industrial cancers in humans, 2-naphthylamine (IX) is certainly one of the more important. The pure amine has been shown to produce bladder tumors when fed to dogs, rats, and rabbits over long periods, and although no bladder tumors were obtained in mice, a substantial number of benign and malignant hepatomas developed. As a matter of fact the true bladder carcinogen appears to be a metabolic product, 2-amino-1-naphthol (X). Conjugates of this compound have been identified in the urine of dogs and of other species following the administration of 2-naphthylamine, and 2-amino-1-naphthol has been shown to be a potent carcinogen when tested directly on the bladder epithelium of the mouse (Bonser, 1943; Bonser, Clayson, and Jull, 1951).



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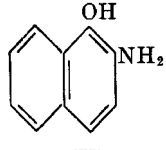
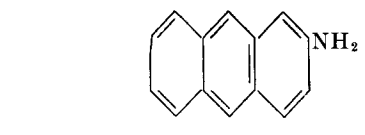


(X)

Many related aromatic amines have been tested for carcinogenic activity, and it has been found that all the active compounds are 2-substituted amines. Several different types of tumor have been induced in rats and in mice with 2-anthramine (XI), but the isomeric anthramines appear to be inactive (Bielschowsky, 1946, 1947). Wilson, DeEds, and Cox (1941) found that 2-acetylaminofluorene (XII) gives tumors in a great variety of organs in rats receiving the compound by mouth, and it was subsequently found that mice are also affected. The acetyl group probably has no biological significance, for 2-aminofluorene is equally carcinogenic. 2-Nitrofluorene is also active, and it seems likely that this compound is reduced *in vivo* to 2-aminofluorene. 2-Acetylaminofluorene is metabolized in part to 2-acetylamino-7-hydroxyfluorene, but this compound failed to give tumors when tested in the same manner as the parent substance (Bielschowsky, 1947). *N*-Dimethyl-2-aminofluorene is much less active than 2-aminofluorene (Bielschowsky and Bielschowsky, 1952).



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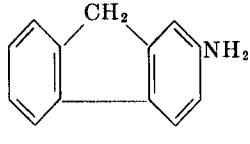
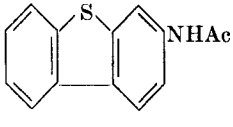


(XII)



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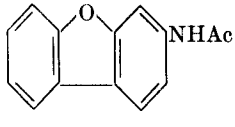

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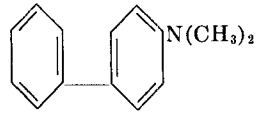
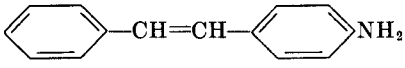
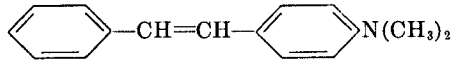


(XV)

3-Acetylaminodibenzothiophen (XIII) and 3-acetylaminodibenzofuran (XIV) have also been tested and shown to be carcinogenic, but the latter compound is somewhat less active than 2-acetylaminofluorene. The nature of the central ring is therefore not of great importance and in this connection it is of interest that 4-dimethylaminodiphenyl (XV) is also carcinogenic. This compound was tested in male rats and gave tumors in the mammary glands, ear duct, liver, and vertebral canal (Miller, Miller, Sandin, and Brown, 1949).



(XVI)



(XVII)

Various aminostilbenes have also been shown to be cancer producing, particularly in rats (Haddow, Harris, Kon, and Roe, 1948). For example,

4-aminostilbene (XVI), 4-dimethylaminostilbene (XVII), and several related compounds, produce tumors in a variety of organs in the rat when administered subcutaneously, or by mouth. Structurally, the active compounds are all closely related to the carcinogenic amino-azo compounds, but this relationship may be largely fortuitous; biologically, they are more closely related to 2-acetylaminofluorene.

The aminostilbenes represent one of the most outstanding successes of the theory associating growth inhibition with carcinogenic activity. These compounds were tested for carcinogenic activity only after they had been shown to be potent tumor inhibitors.

4. Miscellaneous Chemical Carcinogens

A number of other carcinogenic compounds have been discovered more or less accidentally in the course of other biological work, and several additional classes of chemical carcinogen have been found as a result of screening tests of tumor-inhibiting agents.

Ethyl carbamate (urethane) has been shown to produce lung tumors in various strains of mice (Nettleship, Henshaw, and Meyer, 1943), and some other esters of carbamic acid have been shown to have slight activity or none at all (Larsen, 1947). Other hypnotics which have been tested have failed to produce tumors.

The nitrogen mustards, methyl di-(2-chloroethyl)amine and tri-(2-chloroethyl)amine, have also been shown to produce tumors, mostly in the lung, in mice (Boyland and Horning, 1949). Mustard gas or "sulfur mustard" has also been shown to be cancer producing (Heston, 1950).

Hepatomas have been induced in mice with carbon tetrachloride (Edwards, 1941), and with chloroform (Eschenbrenner and Miller, 1945). Liver tumors have been observed in rats following intermittent feeding with alkaloids of *Senecio jacobaea* (Cook, Duffy, and Schoental, 1950). Tannic acid also produces hepatomas and cholangiomas in rats when administered subcutaneously (Korpássey and Mosonyi, 1950).

A number of tumor-inhibiting agents of the cross-linking type have given tumors in experimental animals. These include trimethylolmelamine and certain dimesylglycols (see Boyland, 1952).

Inorganic carcinogens include arsenic, metallic nickel, chromates, salts of zinc, and salts of beryllium. The last named have been shown to produce osteosarcomas in rabbits (Dutra and Largent, 1950).

At the present time, therefore, several hundred chemical compounds belonging to many different chemical classes have been shown to be cancer producing.

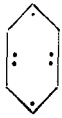
III. Polycyclic Aromatic Hydrocarbons

1. The Structure of Aromatic Compounds

Aromatic compounds have been studied for about a hundred years, and there have been many attempts to assign adequate structural formulas to these substances. Benzene is known to have a hexagonal structure, and as each carbon atom contributes four valency electrons and each hydrogen one, it must have a total of thirty valency electrons. Twelve electrons are involved in the formation of the six carbon-carbon single bonds (σ bonds), and another twelve are required for the six carbon-hydrogen bonds. Six electrons remain, one from each carbon atom, and these must also be involved in some sort of bond formation. If they are grouped in pairs as in (XVIII), the classical Kekulé formula is obtained, and this is normally written as in (XIX). Alternatively, the electrons might be grouped as in (XX), which is equivalent to the Dewar structure (XXI) for benzene. Neither structure can be considered satisfactory for benzene, however, for neither explains its peculiar stability. Moreover, the experimental evidence indicates that all the carbon-carbon bonds in benzene are identical, and that there are no double bonds and no single bonds in the classical sense.

(XVIII)

(XIX)



(XX)

(XXI)

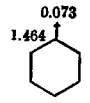
It is only in recent years that the problem of disposing of these six electrons (the aromatic sextet) has been solved by application of quantum mechanics. These electrons are sometimes called π electrons, or "mobile" electrons.

Two quantum mechanical methods have been used, the valence bond method (V.B.), and the method of molecular orbitals (M.O.). An account of these methods has been given in Volume I of this series, and it is therefore unnecessary to outline the principles here (Coulson, 1952).

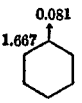
Both methods indicate that there are no carbon-carbon single bonds in benzene, and no carbon-carbon double bonds. All the bonds are found to be identical, and all have "character" intermediate between single and double bonds. Both methods assign *bond orders* to bonds of intermediate character, but different definitions are used, and there is no reason to expect that the numerical values obtained by the two methods for the same bond will agree. It is reasonable to expect, however, that the methods will

agree whether a given bond "A" is more like a double bond than "B," or vice versa. Satisfactory agreement is usually found, but as the two methods are based on different approximations, this is not always the case. In benzene the carbon-carbon bonds are found to have a bond order of 1.464 by the V.B. method and of 1.667 by the M.O. method.

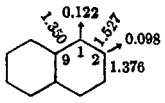
Both methods also assign free valence numbers to the various carbon atoms. These free valence numbers may be considered as relative measures of the unused bonding capacity of the carbon atoms. In benzene (XXII), all the carbon atoms are identical, and all have the same free valence number. Here again, however, it must be emphasized that the two methods do not give the same numerical values.



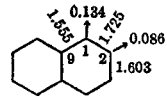
(XXIIa)
(V.B. method)



(XXIIb)
(M.O. method)

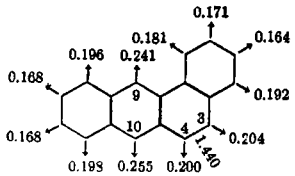


(XXIIIa)
(V.B. method)

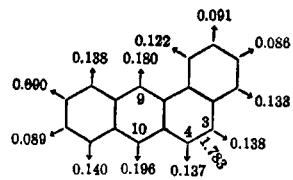


(XXIIIb)
(M.O. method)

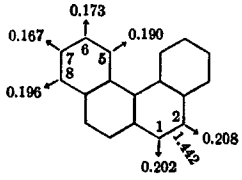
Of course benzene is a perfectly symmetrical molecule, and all the carbon atoms are equivalent; but this is no longer the case with naphthalene (XXIII) and the polycyclic aromatic hydrocarbons. The 1,2 bond in naphthalene has a greater bond order than the 9,1 or the 2,3 bonds, whether this is evaluated by the V.B. or by the M.O. method (A. Pullman, 1947; Coulson and Longuet-Higgins, 1947). Moreover, the 1 position has a greater free valence number than the 2 position; and in most substitution reactions the 1 position is preferentially attacked.



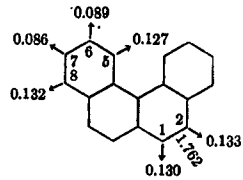
(XXIVa)
(V.B. method)



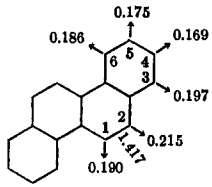
(XXIVb)
(M.O. method)



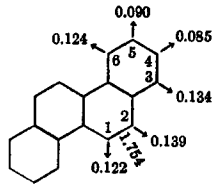
(XXVa)
(V.B. method)



(XXVb)
(M.O. method)



(XXVIa)
(V.B. method)



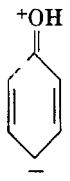
(XXVIb)
(M.O. method)

With the polycyclic aromatic hydrocarbons, the situation is similar. In 1,2-benzanthracene (XXIV), for example, the 10 position has the greatest free valence number, and the 3,4 bond has the greatest bond order. In 3,4-benzphenanthrene (XXV), the 2 position has the greatest free valence number, and the 1,2 bond has the greatest bond order. In chrysene (XXVI), the 2 position has the greatest free valence number and the 1,2 bond has the greatest order (A. Pullman, 1947; Berthier *et al.*, 1948).

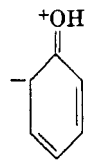
These facts are significant as it has been suggested that one requirement for carcinogenic activity is a phenanthrene type bond having a high order. It is also noteworthy that the positions having the greatest free valence numbers are the ones normally attacked in substitution reactions.

2. Substituents in Aromatic Compounds

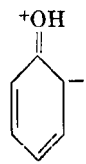
Substituents are of two main classes, those which act as electron donors and increase the electron density of the ring system, especially at the *ortho* and *para* positions, and those which attract electrons and decrease the electron density of the ring system, particularly at the *ortho-para* positions. On account of differences in electronegativity the substituents exert inductive effects; but in most cases the more important effects are those due to conjugation (tautomeric effect, resonance effect). The substituent becomes conjugated with the ring system and additional structures, in which the substituent is linked to the ring system by a double bond, contribute to the resonance hybrid. For example, the three structures (XXVII) contribute to the phenol hybrid, and the three structures (XXVIII) to the hybrid of nitrobenzene.



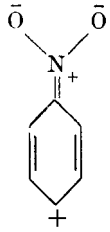
(XXVIIa)



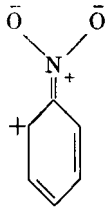
(XXVIIb)



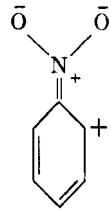
(XXVIIc)



(XXVIIIa)



(XXVIIIb)



(XXVIIIc)

One consequence of this contribution is that the bond linking the substituent to the ring system acquires some double bond character and hence becomes somewhat shorter than a pure single bond of the same type. As a matter of fact the degree of shortening can be used as a measure of the percentage contribution of the ionic structures to the resonance hybrid. The degree of shortening of the bond linking the hydroxy group to the phenyl ring in phenol, for example, indicates that this bond must have 16% double bond character, and the ionic structures therefore contribute 16% to the resonance hybrid of phenol. It is possible to evaluate the approximate increase in electronic charge at the *ortho* and *para* positions caused by a given substituent (B. Pullman, 1948). For a hydroxy group this increase in charge is about 0.053*e* (16% of 0.333*e*, the contribution of the three ionic structures).

The increase or decrease in electronic charge at the various positions in an aromatic ring system can be calculated in several other ways; but all the methods are very approximate, and different methods frequently give values which differ by a factor of 2 or 3.

It must also be remembered that in naphthalene and in polycyclic compounds the different substitution positions do not all have the same conjugating abilities. For example there are seven possible ionic structures which must contribute to the resonance hybrid of a 1-substituted naphthalene and only six similar structures for a 2-substituted naphthalene. It is to be expected, therefore, that a given substituent will be conjugated with the ring system to a greater extent when it is present in the 1 position than when it is in the 2 position, and this has received ample experimental confirmation (Badger, Pearce, and Pettit, 1952).

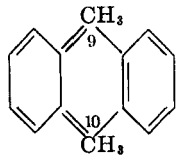
Similar differences in conjugating ability are found in polycyclic compounds. It can be shown that the conjugating ability of a given position is directly related to its free valence number (B. Pullman, 1946), and this generalization is of great utility. Furthermore, there is a linear relationship between the free valence numbers and the bond orders of the linkages joining the substituents to the various positions: the greater the conjugation, the greater the bond order (Daudel, 1950).

Conjugating ability also parallels the self-polarizability, as calculated by the M.O. method (Coulson and Longuet-Higgins, 1948). This provides an additional check, but relatively few self-polarizability values are available for the carcinogenic compounds at present.

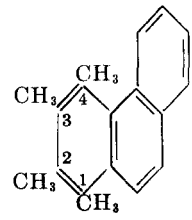
3. General Survey of Polycyclic Carcinogens

1,2,5,6-Dibenzanthracene (I), 3,4-benzpyrene (III), and 20-methylcholanthrene (IV) may all be considered as derivatives of 1,2-benzanthracene, and it was natural that this ring system should be thoroughly investigated. Other ring systems have not been neglected, however, and the tricyclic, tetracyclic, and pentacyclic systems have all been studied.

Among the simpler compounds, anthracene and phenanthrene have been shown to be inactive. In both ring systems, however, the introduction of substituent methyl groups leads to compounds having slight carcinogenic activity. Both 9,10-dimethylanthracene (XXIX) and 1,2,3,4-tetramethylphenanthrene (XXX) have given tumors when tested by application to the skin of mice. These two compounds represent the simplest known chemical carcinogens of the polycyclic type (Kennaway, Kennaway, and Warren, 1942; Badger *et al.*, 1942).



(XXIX)



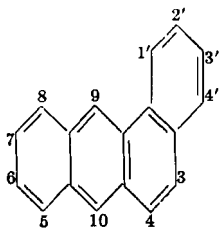
(XXX)

1,2-Benzanthracene (XXXI) seems to be inactive when applied to the skin of mice, but it has slight carcinogenic activity when administered by injection subcutaneously (Steiner and Falk, 1951). Two hepatomas have also been produced in six rats of the Osborne and Mendel strain which were fed 1,2-benzanthracene (White and Eschenbrenner, 1945). Many simple derivatives of 1,2-benzanthracene, however, are active carcinogens, and some di- and trimethyl derivatives are among the most potent of all the known chemical carcinogens (for references see Badger, 1948).

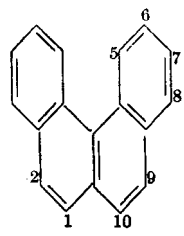
There are twelve possible monomethyl derivatives, and all have been examined, most of them by injection as well as by the skin-painting technic. The 10-methyl, 9-methyl, and 5-methyl derivatives are all moderately potent, the 3-, 4-, 6-, 7-, and 8-methyl derivatives are all weak carcinogens, and the 1′-, 2′-, 3′-, and 4′-methyl derivatives are all inactive.

In general, the dimethyl- and trimethylbenzanthracenes are more active than the monomethyl derivatives from which they are derived. 9,10-Dimethyl-1,2-benzanthracene is a particularly active compound, which has produced skin tumors in mice after an average latent period of only 43 days; and the 5,9- and 5,10-dimethyl derivatives are also very potent carcinogens. The 5,9,10- and 6,9,10-trimethyl derivatives also have very marked carcinogenic activity, especially toward the skin of mice. Cholanthrene can be considered as a 5,10-disubstituted benzanthracene, and 20-methylcholanthrene is a 5,6,10-trisubstituted derivative. Both hydrocarbons are extremely potent carcinogens when tested either by subcutaneous injection, or by the skin-painting technic.

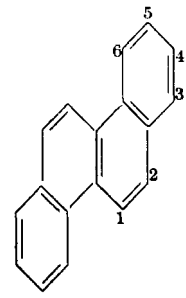
However, all the dimethylbenzanthracenes having a methyl substituent in the benz-ring (positions 1′, 2′, 3′, and 4′) are inactive. For example, the 1′,10-, 2′,6-, 2′,7-, 3′,6-, and 3′,7-dimethyl-1,2-benzanthracenes have all failed to produce tumors in mice. Further reference will be made to this fact in a subsequent section.



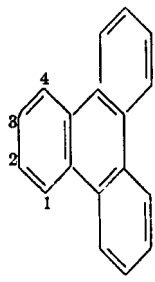
(XXXI)



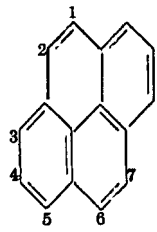
(XXXII)



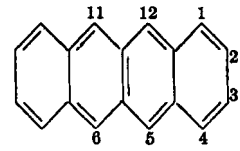
(XXXIII)



(XXXIV)



(XXXV)



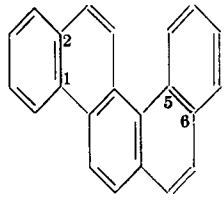
(XXXVI)

3,4-Benzphenanthrene (XXXII) is another important ring system. The parent compound itself gave tumors of the skin in a relatively large proportion of the mice treated, but only after a latent period of the order of 15 months, so it must be considered as a very weak carcinogen. The 6-, 7-, and 8-methyl derivatives are also weak carcinogens, but 1-methyl-3,4-benzphenanthrene has moderate activity and 2-methyl-3,4-benzphenanthrene is a relatively potent carcinogen when tested on the skin of mice. It is a curious fact that the 3,4-benzphenanthrenes are all much less active when administered by injection. Of all the methyl derivatives only the 2- and the 6- have given tumors by this method.

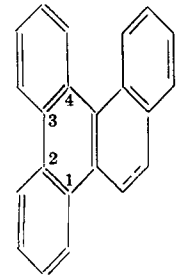
Chrysene (XXXIII) seems to be inactive when tested on the skin of mice, but there is some evidence that it has very weak activity when administered by injection (Steiner and Falk, 1951). Relatively few derivatives of this ring system have been tested, but here again it has been shown that methyl substituents in "favorable" positions greatly increase the carcinogenic activity. Thus 1-methylchrysene is quite active in the production of sarcomas, and 1,2-dimethylchrysene is moderately active by the skin-painting technic.

Triphenylene (XXXIV), pyrene (XXXV), and naphthacene (XXXVI) have all been shown to be inactive, and all the simple derivatives that have been tested have also failed to induce tumors.

Of the pentacyclic ring systems, 1,2,5,6-dibenzanthracene and 3,4-benzpyrene were shown to be active in the early studies of the polycyclic compounds, and this naturally led to the examination of the thirteen other pentacyclic compounds. 1,2,5,6-Dibenzphenanthrene (XXXVII) and 1,2,3,4-dibenzphenanthrene (XXXVIII) were found to be moderately active, and 1,2,7,8-dibenzanthracene was found to be a weak carcinogen; but all the other compounds of this type failed to produce tumors.



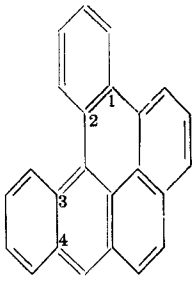
(XXXVII)



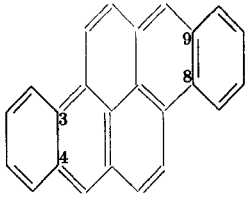
(XXXVIII)

It is noteworthy that whereas methyl substitution in tetracyclic compounds often develops or increases the carcinogenic activity, the results of similar substitution in pentacyclic compounds are somewhat irregular.

Thus 9-methyl-1,2,5,6-dibenzanthracene is more active than the parent ring system, but 9,10-dimethyl-1,2,5,6,-dibenzanthracene is much less so. On the other hand, 9,10-dimethyl-1,2,7,8-dibenzanthracene is much more active than the unsubstituted compound. Again, although 1,2,3,4-dibenzphenanthrene is an active carcinogen, its 9-methyl, and 10-methyl derivatives appear to be inactive. Several methyl derivatives of 3,4-benzpyrene have been tested. The 2′- and 3′-methyl derivatives are inactive, the 4′- and 6-methyl derivatives appear to be less active than the parent compound, and the 5-methyl and 9-methyl derivatives have been shown to possess approximately the same activity as the parent compound.



(XXXIX)



(XL)

Among the more complex polycyclic compounds it may be mentioned that 1,2,3,4-dibenzpyrene (XXXIX) and 3,4,8,9-dibenzpyrene (XL) have both given tumors. These are the largest known chemical carcinogens.

4. The "Favorable" Positions for Methyl Substitution

It has been shown that the introduction of one or more methyl substituents into certain "favorable" positions often develops or increases the carcinogenic activity of a polycyclic aromatic hydrocarbon. It is also known that the various substitution positions in a hydrocarbon such as 1,2-benzanthracene vary considerably in conjugating ability, and it is interesting to enquire whether the two facts are related.

For 1,2-benzanthracene, the conjugating abilities are given by the self-polarizabilities, by the V.B. free valence numbers, and by the M.O. free valence numbers. It has been shown that there is an approximate linear relationship between the polarizabilities and both sets of free valence numbers, so that either set of values may be used as indices of the conjugating abilities (Badger, Pearce, and Pettit, 1952).

Substitution in the angular ring of 1,2-benzanthracene invariably inactivates the molecule as far as carcinogenesis is concerned (Badger, 1948). Steric factors may be involved here (see later), and these positions may therefore be omitted for the moment. Considering the eight other

TABLE I
Conjugating Abilities of Various Substitution Positions in 1,2-Benzanthracene and Carcinogenic Activities of Corresponding Methylbenzanthracenes

Position	Index of Conjugating Ability			Carcinogenic Activity of Methylbenzanthracenes[d]	
	Self-polarizability[a] (M.O. method)	Free-valence Number[b] (M.O. method)	Free-valence Number[c] (V.B. method)	Skin	Subcutaneous Tissue
6	0.409	0.089	0.168	+	
7	0.410	0.090	0.168	+	+
4	0.441	0.137	0.200	+	++
8	0.449	0.138	0.196	+	0
3	0.449	0.138	0.204	+	++
5	0.452	0.140	0.198	++	++
9	0.495	0.180	0.241	++	+++
10	0.513	0.196	0.255	+++	++++

[a] Badger, Pearce, and Pettit (1952).
[b] Berthier *et al.* (1948). Values adjusted for $F_{max} = 3 + \sqrt{2}$.
[c] A. Pullman (1947).
[d] Badger (1948).

substitution positions (Table I) it quickly appears that the 5, 9, and 10 positions have the greatest conjugating abilities, and it is significant that methyl substitution at these positions produces the most active carcinogens. All the possible dimethylbenzanthracenes involving these positions are very active carcinogens, and 5,9,10-trimethyl-1,2-benzanthracene is one of the most potent compounds known for the skin of mice.

In 3,4-benzphenanthrene, both sets of free valence numbers indicate that the 2 position has the greatest conjugating ability; and it is significant that the parent compound is attacked at this position in substitution reactions. The 1 position also has high conjugating ability. As indicated in Table II, 2-methyl-3,4-benzphenanthrene is a potent carcinogen, and 1-methyl-3,4-benzphenanthrene has moderate activity against the skin of mice. Here again, therefore, it seems that the "favorable" positions are the positions of greatest conjugating power. In this connection Newman and Kosak (1949) have pointed out that carcinogenic activity appears when a methyl group is introduced at a position of high chemical reactivity.

For chrysene, the two sets of free valence numbers both indicate that the 2 position has the greatest conjugating power, and this is also supported by the fact that substitution reactions also involve this position predominantly (Berthier *et al.*, 1948; A. Pullman, 1947; Newman and Cathcart, 1940). Only three of the six possible monomethylchrysenes have

TABLE II
Conjugating Abilities of Various Substitution Positions in 3,4-Benzphenanthrene and Carcinogenic Activities of Corresponding Methylbenzphenanthrenes

Position	Index of Conjugating Ability		Carcinogenic Activity of Methylbenzphenanthrenes[c]	
	Free valence Number[a] (M.O. method)	Free valence Number[b] (V.B. method)	Skin	Subcutaneous tissue
7	0.086	0.167	+	0
6	0.089	0.173	+	+
5	0.127	0.190		
8	0.132	0.196	+	0
1	0.130	0.202	++	0
2	0.133	0.208	+++	+

[a] Berthier *et al.* (1948). Values adjusted for $F_{max} = 3 + \sqrt{2}$.
[b] A. Pullman (1947).
[c] Badger (1948).

been tested for carcinogenic activity, and these only by subcutaneous injection. In this case, however, although the 2-methyl derivative is active, 1-methylchrysene seems to be the most potent derivative (Badger, 1948). It is interesting that 1,2-dimethylchrysene is a potent carcinogen to the skin of mice, and it is to be hoped that the monomethylchrysenes will all be tested by the skin-painting technic.

On the whole, therefore, the evidence indicates that the "favorable" positions in potentially carcinogenic ring systems are the positions having the greatest conjugating abilities. Methyl substituents in such positions donate more electronic charge to the ring system than when they are present in positions having smaller conjugating abilities, and it is reasonable to conclude that carcinogenic activity is intimately connected with the electronic configuration and electron density of the hydrocarbon.

The study of the ultraviolet absorption spectra of substituted compounds also provides some information on this point. All the 1,2-benzanthracenes, for example, have similar absorption spectra, but alkyl substitution is found to shift the absorption bands to longer wavelengths. Absorption spectra are rather complicated and it is not to be expected that any simple relationships will hold in an accurate way; but it can easily be shown that the magnitude of the bathochromic shift is directly related to the conjugating ability of the substitution position. Of the monomethyl derivatives, for example, the largest shifts are produced by 9-methyl- and 10-methyl-1,2-benzanthracenes (Badger, Pearce, and Pettit, 1952). This is the explanation of the fact that there is an approximate correlation between the carcinogenic activities of a series of substituted benzanthracenes

and the position of the most intense absorption band, as was first observed by Jones (1940).

5. The Effect of Other Substituents

It has been shown that although 1,2-benzanthracene is an inactive, or very feeble active compound, a single methyl substituent in a "favorable" position gives a potent carcinogen. It is therefore of interest to examine the effects of other conjugating substituents and to compare the effects of electron-donating and electron-attracting groups.

The effects of higher alkyl groups have received a fair amount of attention. The higher alkyl groups also exhibit hyperconjugation and act as electron donors to the ring system. Nevertheless, it has been established for several series of alkyl derivatives that carcinogenic activity decreases with increasing length of carbon chain (Badger, 1948). The 5-*n*-alkyl-1,2-benzanthracenes, for example, are progressively less active toward the skin of mice as the series is ascended as far as the *n*-heptyl derivative; and when tested by subcutaneous injection, the higher members (above propyl) of the series are all inactive. Similarly, among the 10-*n*-alkyl-1,2-benzanthracenes, activity rapidly falls off as the series is ascended, and the *n*-propyl, *n*-butyl, and *n*-amyl derivatives are all inactive. Again, 20-*t*-butylcholanthrene and 20-isopropylcholanthrene are much less active than 20-methylcholanthrene. This decrease in carcinogenic activity with increasing chain length cannot be due to electronic effects; but it does seem likely that the increasing size of the alkyl group is responsible for the effect. Such steric effects are considered in a later section.

The effects of other substituents have also been chiefly studied in 1,2-benzanthracene. More than twenty 10-substituted derivatives have been synthesized, mostly by direct substitution, or by simple transformations from substitution products. A few of these (e.g., 10-hydroxy-1,2-benzanthracene) may possibly be excreted too rapidly for any tumor-producing activity to become apparent. Nevertheless, many of these derivatives are active (Table III) and brief consideration of the nature of the substituents indicates that both electron-donating and electron-attracting substituents can transform benzanthracene into a relatively active carcinogenic compound. Thus the 10-methyl-, 10-amino-, 10-mercapto-, and 10-methoxy-1,2-benzanthracenes are all cancer producing, and all these substituents are known to be electron donating. On the other hand, 10-cyano- and 10-formyl-1,2-benzanthracenes are also carcinogenic although these substituents are electron attracting. In this connection it is interesting that the 5-bromo and 5-cyano derivatives of 9,10-dimethyl-1,2-benzanthracene have approximately the same activity as the parent dimethylbenzanthracene, and that 9-methyl-10-cyano-1,2-benzanthracene is a

particularly potent carcinogen. It is curious that although 10-bromo-1,2-benzanthracene is inactive, 10-chloro-1,2-benzanthracene does produce tumors.

TABLE III
10-Substituted 1,2-Benzanthracenes[a,b]

Substituent in the 10 Position	Carcinogenic Activity	
	Skin	Subcutaneous Tissue
$-CH_3$	+++	++++
$-CH_2CN$		+
$-CH_2Cl$		0
$-CH_2SH$		+
$-CH_2OH$	++	+++
$-CH_2OCH_3$		0
$-CH_2OCOCH_3$	++	+++
$-CH_2OCH_2CH_3$	+	0
$-CH_2NMe_2$		0
$-CH_2NEt_2$		0
$-CH_2COOH$		0
$-CH_2COOCH_3$		+
$-OH$		0
$-OCH_3$		++
$-NO_2$		0
$-NH_2$		+
$-CN$	+	++
$-SH$		+
$-NCO$		+
$-CHO$		++
$-CHOH.CH_3$		0
$-COCH_3$	0	
$-Cl$	++	
$-Br$	0	

[a] Badger (1948).
[b] Lacassagne *et al.* (1948).

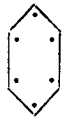
The effects of various substituents in other ring systems have not been extensively studied, but there is no reason to doubt that the effects will be similar to those observed for 1,2-benzanthracene. For example, 5-methyl-3,4-benzpyrene and 3,4-benzpyrene-5-aldehyde are both potent carcinogens, and 5-hydroxy-3,4-benzpyrene is inactive (Shear, Leiter, and Perrault, 1940).

This brief survey clearly indicates that although the electronic character of the substituent may well be of importance, it cannot be the whole story, and other effects must also operate.

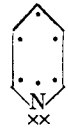
6. *Heterocyclic Analogs of Carcinogenic Hydrocarbons*

It has often been suggested that the general shape and size of the molecule is of some importance in determining carcinogenic activity, and it is therefore of interest to examine heterocyclic analogues of known carcinogenic hydrocarbons.

Electronically, the heterocyclic ring systems resemble benzene (XLI) very closely; but there are several important differences. The hetero atoms are more electronegative than carbon, and they do not have the same number of valency electrons. In pyridine (XLII), each carbon atom contributes one electron to the common pool of mobile electrons, and the nitrogen atom also contributes one electron to complete the aromatic sextet. Two more electrons from the nitrogen atom are involved in the formation of the σ bonds with the adjacent carbon atoms. Unlike carbon, however, nitrogen has five valency electrons, so that in pyridine two electrons are not involved in bond formation. These constitute the "lone pair," and are responsible for the fact that pyridine is basic and readily forms salts.



(XLI)



(XLII)

The most important five-membered heterocyclic ring systems are pyrrole (XLIII), thiophen (XLIV), and furan (XLV). In these molecules each hetero atom contributes two electrons to the common pool of π electrons to form the aromatic sextet. The hetero atom, with its pair of electrons, may therefore be considered as replacing one of the C=C groups, with its pair of π electrons, in benzene. As a matter of fact the resemblance between the "lone pair" of sulfur and the two π electrons of ethylene is very striking indeed. Walsh (1948) has pointed out that the potential required to ionize one of the sulfur electrons is 10.47, as against 10.50 volts for the ionization of a π electron in ethylene. It is also noteworthy that in pyrrole, all the valency electrons of the nitrogen atom are involved in bond formation so that no "lone pair" of electrons is available for salt formation. Pyrrole cannot form salts except at the expense of its aromatic character.

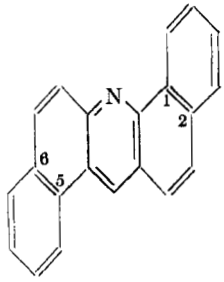
(XLIII)

(XLIV)

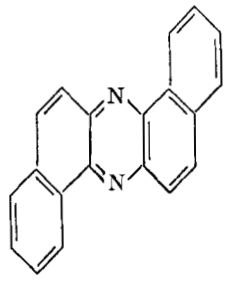
(XLV)

All the heterocyclic compounds are therefore very similar to benzene and they retain all the essential features of aromaticity. It is not surprising that these ring systems can sometimes replace benzene in biologically active molecules.

Condensed heterocyclic ring systems also resemble their carbocyclic analogs very closely indeed, and many polycyclic heterocyclic compounds are now known to possess carcinogenic properties. Structural similarity is not the only requirement for carcinogenic activity, however, for although 1,2,5,6-dibenzacridine (XLVI) is slightly active, 1,2,5,6-dibenzphenazine (XLVII) is inactive.

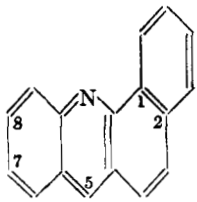


(XLVI)

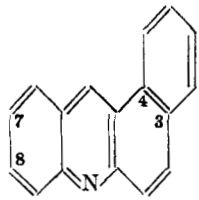


(XLVII)

The methylbenzacridines have been extensively studied in recent years and these compounds provide an excellent example of the fact that the position of the hetero atom can be all important (Lacassagne *et al.*, 1946, 1947). 1,2-Benzacridine (XLVIII) is inactive, but by suitable methyl substitution marked carcinogenic activity is developed. For example, 5,7-dimethyl-1,2-benzacridine and 5,8-dimethyl-1,2-benzacridine are very potent carcinogens, particularly when applied to the skin. With 3,4-benzacridine (XLIX), however, it is much more difficult to develop carcinogenic activity by methyl substitution. 5,7-Dimethyl-3,4-benzacridine is inactive, and 5,8-dimethyl-3,4-benzacridine is only a very weak carcinogen. Carcinogenic activity of a high order can only be developed by *three* methyl groups in favorable positions. 5,7,9-Trimethyl-3,4-benzacridine is a fairly potent carcinogen to the skin of mice, although it is inactive when administered by injection.

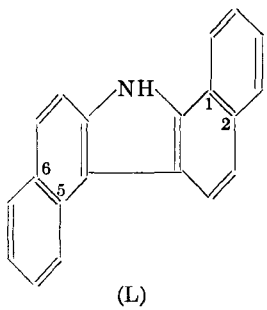


(XLVIII)

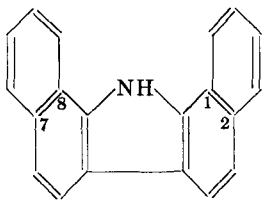


(XLIX)

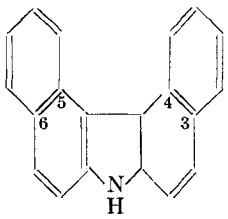
Several derivatives of five-membered heterocyclic ring systems have also been tested, and of these the dibenzcarbazoles are of special interest. Boyland and Brues (1937) found that both 1,2,5,6-dibenzcarbazole (L) and 3,4,5,6-dibenzcarbazole (LII) are active when tested by application to the skin of mice, and 1,2,7,8-dibenzcarbazole (LI) proved to be a weak carcinogen. In addition to the local tumors at the site of application, the mice treated with 3,4,5,6-dibenzcarbazole showed malignant changes in the liver, and further reference will be made to this observation in the section on azonaphthalenes.



(L)



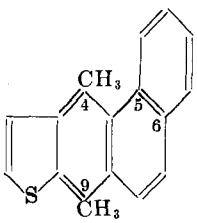
(LI)



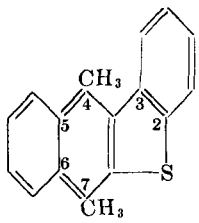
(LII)

Two thiophen analogs of the potent carcinogen, 9,10-dimethyl-1,2-benzanthracene have been tested. 4,9-Dimethyl-5,6-benzthiophanthrene (LIII) was found to have activity of the same order as that of the hydrocarbon, and produced tumors in mice after an average latent period of only 116 days (Dunlap and Warren, 1941). The isomeric compound, 4,7-dimethyl-2,3,5,6-dibenzthionaphthene (LIV), was found to be only slightly active when tested on the skin of mice, and inactive when administered by subcutaneous injection (Tilak, 1951). This observation is of considerable interest as the latter compound does not possess a phenanthrene skeleton. In this compound the essential feature of the phenanthrene ring system, the reactive 9,10 bond, is replaced by a sulfur atom.

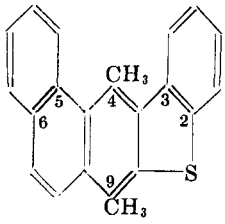
In this connection it may be significant that 4,9-dimethyl-2,3,5,6-dibenzthiophanthrene (LV) and 4,9-dimethyl-2,3,7,8-dibenzthiophan-



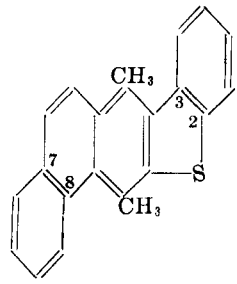
(LIII)



(LIV)



(LV)

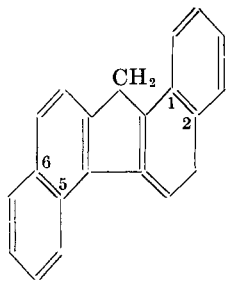


(LVI)

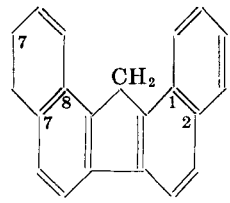
threne (LVI) are particularly potent carcinogens both to the skin of mice, and to subcutaneous tissue (Tilak, 1951). Both these compounds possess a phenanthrene ring system.

7. Fluorene Derivatives

1,2,5,6-Dibenzfluorene (LVII) and 1,2,7,8-dibenzfluorene (LVIII) have both been shown to have feeble carcinogenic activity when tested on the skin of mice; but the 3,4,5,6 derivative appears to be inactive.



(LVII)



(LVIII)

These observations are of some importance for although these substances may be said to have roughly the same molecular dimensions as the related dibenzanthracenes and dibenzcarbazoles, they are very different electronically. In the dibenzcarbazoles the nitrogen atom contributes two electrons to the common pool of mobile electrons. In the fluorenes, however, the central carbon atom is *saturated* and cannot contribute to the pool of mobile electrons, and the dibenzfluorenes are therefore more akin to the dinaphthyls than to the dibenzcarbazoles.

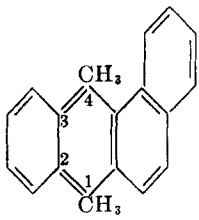
8. The Phenanthrene Type Double Bond

Although phenanthrene itself is not cancer producing, many of its derivatives are active. Hewett (1940) has pointed out that nearly all the potent carcinogens can be considered as phenanthrene derivatives having

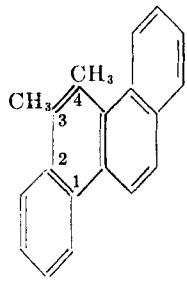
additional benzene rings and/or methyl groups at three or four of the positions 1, 2, 3, and 4.

1,2-Benzanthracene can be considered as 2,3-benzphenanthrene, and if methyl groups are introduced at the 1 or 4 positions, carcinogenic hydrocarbons are obtained. For example, 1-methyl-2,3-benzphenanthrene is 10-methyl-1,2-benzanthracene; and 1,4-dimethyl-2,3-benzphenanthrene is 9,10-dimethyl-1,2-benzanthracene (LIX). Both hydrocarbons are very potent carcinogens.

Similarly, chrysene can be considered as 1,2-benzphenanthrene and, if methyl groups are introduced at the 3 or 4 positions, carcinogenic activity is obtained. 3,4-Dimethyl-1,2-benzphenanthrene, or 1,2-dimethylchrysene (LX), for example, is a moderately active carcinogen.

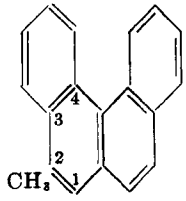


(LIX)*

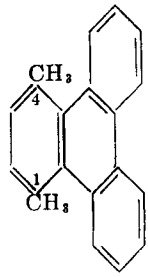


(LX)*

2-Methyl-3,4-benzphenanthrene (LXI) is also a potent carcinogen, and this hydrocarbon can be considered as a phenanthrene derivative substituted at the 2, 3, and 4 positions. Hewett and Martin (1940) therefore prepared 1,2,3,4-tetramethylphenanthrene, and this compound was also shown to have slight carcinogenic activity.



(LXI)



(LXII)

The most striking feature of the phenanthrene molecule is the reactive 9,10 double bond, and it is therefore significant that 1,4-dimethyltri-

* Phenanthrene numbering is used in these formulas.

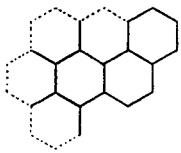
phenylene (LXII), in which this double bond is absent, is inactive. 1,2,3,4-Dibenzanthracene and 9,10-dimethyl-1,2,3,4-dibenzanthracene are also inactive, and neither compound has a phenanthrene type double bond.

Robinson (1946) has suggested that the essential feature in most of the polycyclic carcinogens is a phenanthrene type double bond which is "activated" by suitable substitution, or by additional benzene rings. The condensed thiophen derivatives already discussed were prepared to test this hypothesis. It will be recalled that replacement of the phenanthrene type double bond in 9,10-dimethyl-1,2-benzanthracene by a sulfur atom, as in 4,7-dimethyl-2,3,5,6-dibenzthionaphthene (LIV) resulted in a very great loss of activity, although the introduction of a sulfur atom at another site in the molecule (LIII) did not have such an effect. Moreover, reintroduction of a phenanthrene type double bond by the addition of a further benzene ring again gave very potent carcinogenic compounds (LV, LVI).

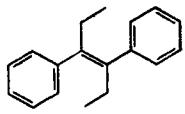
The phenanthrene type double bond does, therefore, seem to have some significance, and it is of interest in this connection that 6,7-dihydro-20-methylcholanthrene (in which the phenanthrene type double bond is reduced) is inactive as a carcinogen.

9. The Influence of Molecular Shape and Size

Experimental evidence is ample that there is an optimum degree of molecular complexity for carcinogenic activity, and it seems that the molecular shape may also be a factor. Nearly all the potent carcinogens can be portrayed by the attached structure (LXIII) in which the dotted lines represent rings or groups which may or may not be present in the active molecule. Moreover, the "open" analog of 3,4-benzpyrene, α-ethyl-β-*sec*butylstilbene (LXIV), is a weak carcinogen (Dodds, Lawson, and Williams, 1941), and it is difficult to escape the conclusion that the shape and size of this molecule is of significance here.



(LXIII)



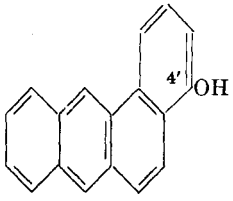
(LXIV)

Since this is so, it is not unreasonable to suggest that the carcinogenic molecule may enter into some form of complex with a cellular component, and that the shape and size of the molecule may play an important part in this complex formation. Bergmann (1942) has suggested that the car-

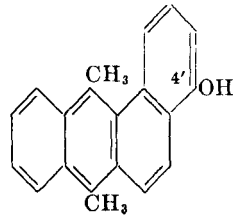
cinogens may be adsorbed by a cellular "receptor" possessing a definite adsorption area, and that this sets an upper limit for the dimensions of the active molecule. A lower limit is given by the decrease in adsorbability with decreasing size of the molecule.

It was an essential feature of the original Bergmann hypothesis that molecules can be inactivated by additions which hinder their proper adsorption. It might be supposed that by increasing the size of a substituent group complex formation would be inhibited. The evidence is not inconsistent with such a view, for the higher alkyl derivatives of 1,2-benzanthracene are all much less active than the corresponding methyl derivatives (Badger, 1948). It might be supposed that adsorption and complex formation would also be inhibited by alterations which involve the destruction of the coplanarity of the molecule. Here again the experimental evidence is not inconsistent with such a view. Such distortions of the molecules occur on hydrogenation, and in most cases it has been found that partial hydrogenation does destroy carcinogenic activity (Badger, 1948).

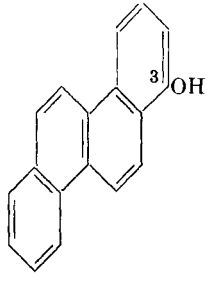
Complex formation is also supported by the fact that the various carcinogenic hydrocarbons all seem to metabolize to hydroxy derivatives in which the hydroxy groups occupy comparable positions. 1,2-Benzanthracene is metabolized to the 4′ derivative (LXV) and 9,10-dimethyl-1,2-benzanthracene is likewise metabolized to the 4′ derivative (LXVI). The 3 position in chrysene is comparable to the 4′ position in 1,2-benzan-



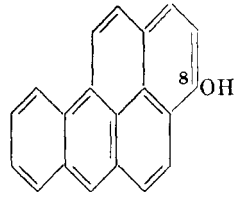
(LXV)



(LXVI)



(LXVII)



(LXVIII)

thracene, and it has been shown that chrysene is metabolized to 3-chrysenol (LXVII). The 8 position in 3,4-benzpyrene is also comparable to the 4′ position in 1,2-benzanthracene, and it is known that 3,4-benzpyrene is metabolized to 8-hydroxy-3,4-benzpyrene (LXVIII) in rats and mice.

All these results are consistent with the view that the molecule is adsorbed in a specific fashion and that metabolic hydroxylation takes place at an exposed position. This might well be the explanation for the fact that all the methylbenzanthracenes with methyl groups in the benz-ring are inactive: methyl substitution in this ring might interfere with the proper formation of the complex. On the other hand, 3,4-benzpyrenes substituted in positions 8, 9, or 10 should also be inactive, but 9-methyl-3,4-benzpyrene is a very potent carcinogen, and 8-methoxy-3,4-benzpyrene also produces multiple skin tumors in a high percentage of mice treated after a relatively short latent period.

Additional evidence favoring complex formation has been provided by a number of experiments involving the inhibition of carcinogenic activity. Lacassagne, Buu-Hoï, and Rudali (1945) made repeated application on the skin of mice with solutions containing a mixture of two hydrocarbons of similar molecular configuration. One component of the mixture was a potent carcinogen, the other was either inactive or only very feebly active. The mixtures used were 1,2,5,6-dibenzfluorene and 20-methylcholanthrene, chrysene and 20-methylcholanthrene, and 1,2,-5,6-dibenzacridine and 1,2,5,6-dibenzanthracene. In each case tumors were produced more slowly than when the potent hydrocarbon alone, at the same concentration, was applied. Strangely enough no such inhibition of the action of 20-methylcholanthrene was produced by admixture with a number of benzacridines. The inhibition of 9,10-dimethyl-1,2-benzanthracene skin carcinogenesis with several polycyclic hydrocarbons has been demonstrated (Hill, *et al.*, 1951); and it has also been shown that 1,2-benzanthracene inhibits the development of sarcomas following the subcutaneous injection of 1,2,5,6-dibenzanthracene (Steiner and Falk, 1951).

The most reasonable explanation of this inhibition is that the two substances, having analogous structures, penetrate into the same cells and form complexes with some cellular component. Each inactive or very weakly active molecule would hinder by its presence the fixation of a molecule of the potent carcinogen and thereby delay the appearance of tumors. The interesting conclusion, however, is that complex formation of itself is not sufficient to bring about malignant transformations. In order to inhibit the action of the potent carcinogens, the inactive compounds must be adsorbed, and yet they do not bring about carcinogenesis.

Bergmann (1942) suggested that the molecule is adsorbed as a whole and that its activity is determined by its shape and size. In recent years, however, it has been suggested that complex formation takes place via the phenanthrene type double bond.

At present the nature of the complex is entirely hypothetical, but Miller (1951) has recently found that 3,4-benzpyrene is firmly bound to a protein in the epidermal fraction of mouse skin.

Aromatic compounds form complexes with a wide variety of agents such as picric acid, s-trinitrobenzene, trinitrofluorenone, aluminum chloride, stannic chloride, and antimony pentachloride. The essential feature seems to be that the complexing agent must contain a number of electron-attracting groups (nitro groups, chloro groups, etc.). All aromatic compounds do not form complexes of equal stability with the same reagent, however. Molecular complexity is important, and molecules such as anthracene and benzanthracene form more stable picrates than those formed from benzene or naphthalene. Moreover, methyl substitution usually enhances the ease of formation and the stability of picrates, and, as has already been shown, methyl substitution generally increases the carcinogenic activity. The most stable picrates are usually deeper in color than the less stable complexes. It is therefore interesting that 9,10-dimethyl-1,2-benzanthracene, 20-methylcholanthrene, 3,4-benzpyrene, and some other potent carcinogens, all form very dark chocolate brown or purplish black picrates. The feebly active or inactive methyl- and dimethylbenzanthracenes, however, give bright red picrates.

It is also of interest that complex formation with picric acid and similar components can be inhibited by steric hindrance. Any substituent which destroys the coplanarity of the molecule hinders complex formation, or causes the complex to form in other than a 1:1 ratio (Orchin, 1951).

Of course it must not be supposed that the carcinogenic molecules form complexes *in vivo* with a component of the above type. Nevertheless the properties which govern picrate formation may well be identical, or almost identical, with those which control the formation of a complex with the cellular component. The actual receptor may possibly be a nucleic acid, and in this connection it is interesting that the presence of purines increases the solubility of aromatic hydrocarbons in water (Brock, Druckrey, and Hamperl, 1938; Weil-Malherbe, 1946). Moreover purines have been found to form complexes with polycyclic aromatic compounds in benzene solution.

10. The Pullman-Daudel Theory

Using the valence-bond method, it is possible to calculate the molecular diagrams for all the simple aromatic hydrocarbons. When this is done

for the tricyclic and tetracyclic hydrocarbons it is found that the potentially carcinogenic ring systems (1,2-benzanthracene, 3,4-benzphenanthrene, chrysene) all have a phenanthrene type bond with a particularly high electron density or bondorder (see pp. 81–2). Other ring systems which are not potentially carcinogenic (triphenylene, naphthacene) do not possess a region having such a high electron density or bond order. Methyl substitution at any part of a potentially carcinogenic ring systen would be expected to increase the electron density at the phenanthrene type bond by a variable amount depending on the position of substitution.

A number of French theoretical chemists (A. Pullman, 1947; Pullman and Pullman, 1946; Daudel, 1948; Daudel and Daudel, 1950) have suggested that carcinogenic activity is associated with an optimum charge of π electrons on the phenanthrene type bond (called the K region; K for Krebs). It was suggested that there is a certain critical value for this charge, below which carcinogenic activity does not occur, and that there is likewise an upper limit. The essence of the theory is that methyl groups act as slight electron donors to a potentially carcinogenic ring system (such as benzanthracene) and thereby increase the electron density of the K region to a value above the critical lower limit. On the other hand, an annular nitrogen atom (as in benzacridine) decreases the charge on the K region by an amount which is dependent on its position in the ring system. Thus an annular nitrogen atom might be compensated for by the presence of one or more methyl groups. Excessive methyl substitution, however, might increase the electron density until it exceeds the upper limit and hence inactivate, or reduce the activity of the molecule.

A. Pullman (1947) evaluated the total charge of π electrons at the K region by summing the two free valence numbers (as calculated by the V.B. method) and the *charge de liaison*. Evaluated in this way, 3,4-benzphenanthrene has a K region with a charge of 1.293*e*, phenanthrene of 1.291*e*, 1,2-benzanthracene of 1.283*e*, and chrysene of 1.272*e*. On the other hand, anthracene has no bond with an electron density greater than 1.259*e*, naphthacene 1.258*e*, and triphenylene 1.260*e*.

The effects of methyl substituents and of annular nitrogen atoms were estimated by a very approximate method and the electron densities for a series of methylbenzanthracenes, methylbenzphenanthrenes, methyl-1,2-benzacridines, and methyl-3,4-benzacridines were evaluated.

Within these series an excellent correlation between electron density and carcinogenic activity was found. The critical charge on the bond, below which the compounds are not carcinogenic, was found to be about 1.290*e*. Thus 1,2-benzanthracene with a charge of 1.283*e* is inactive or only very feebly active, but 3,4-benzphenanthrene with a charge of 1.293*e* is a weak carcinogen. Methyl substitution in the 10 position of 1,2-benz-

anthracene was estimated to increase the charge to 1.306*e*, and this compound is a potent carcinogen. Dimethyl substitution was estimated to increase the charge considerably, the effects of the methyl groups being additive, and these compounds are generally potent carcinogens.

Perhaps the greatest success for the theory was its ability to explain the marked difference in the carcinogenic activities of the methyl-1,2-benzacridines as opposed to the methyl-3,4-benzacridines. The latter are, in general, much less active than the former. This is explained by the fact that although the electronegative nitrogen deactivates the K region in both series, its influence is much greater in the 3,4-benzacridines than in the 1,2-benzacridines. According to A. Pullman (1947), the charge on the K region of 3,4-benzacridine is 1.260*e*, and that on the K region of 1,2-benzacridine is 1.270*e*.

There are several exceptions to this simple theory, however. As has already been emphasized, the methylbenzanthracenes having a substituent in the benz-ring are all inactive although activity would be expected in this group. Moreover, it has been demonstrated that both electron-attracting and electron-donating substituents transform 1,2-benzanthracene into an active carcinogen. It is difficult to accommodate the fact that 10-cyano- and 10-formyl-1,2-benzanthracenes are active cancer-producing substances within the framework of the theory (Badger, 1948). The carcinogenic dibenzfluorenes must also be considered exceptions to the theory, as these substances cannot have a pronounced density of electrons at the K region.

An additional criticism of the theory is that it is too approximate. With substituted compounds the total charge on the K region was obtained by adding the contribution of the substituent to the value for the total charge calculated for the unsubstituted hydrocarbon. This treatment neglects the fact that methyl substituents may also affect the mobile bond order or *charge de liaison*, and it is calculated that the mobile bond order or *charge de liaison* is sometimes *decreased* by methyl substitution (Daudel, 1948). When these adjustments are made, it turns out that the correlation between the charge on the K region and the carcinogenic activity is not nearly so good as before. As a matter of fact it is unlikely that these "rigorous" calculations give a true picture. According to this treatment 10-methyl-1,2-benzanthracene has a *smaller* density of electrons on the K region than the unsubstituted hydrocarbon, and this seems entirely unreasonable.

In view of these difficulties it has been suggested that the most satisfactory method is to compare the carcinogenic activity with the "excess charge" on the K region caused by the methyl substitution. Assuming that the effect of an annular nitrogen atom is equal and opposite to that

TABLE IV

Excess Charge on the K Position Caused by Methyl Substitution or an N Atom at Various Positions[a]

Compound	Position									
1,2-Benzacridine (XLVIII)	6	7	8	9	—	5				
3,4-Benzacridine (XLIX)	9	8	7	6	5	—				
1,2-Benzanthracene (XXXI)	5	6	7	8	9	10	1′	2′	3′	4′
Nature of Substituent	**Excess Charge**									
CH_3	0.016	0.013	0.014	0.010	0.016	0.027	0.015	0.021	0.018	0.025
N-Hetero atom	−0.016	−0.013	−0.014	−0.010	−0.016	−0.027	−0.015	−0.021	−0.018	−0.025

[a] Daudel (1948).

of a methyl substituent in the same position, it is also possible to evaluate the "excess charge" on the K region for heterocyclic compounds. Table IV has been constructed giving the effects of methyl groups or of a nitrogen atom at each position of the benzanthracene ring system (Daudel, 1948), and it is possible to evaluate the excess charges on the K region for nearly all the known methylbenzanthracenes and methylbenzacridines. These are given in Table V; but derivatives having substituents in the

TABLE V

Excess Charge on K Region (V.B. Method) and Carcinogenic Activity

	Excess Charge on K Region	Carcinogenic Activity[a]	
		Skin	Subcutaneous Tissue
3,4-Benzacridine	−0.027*e*	0	
1,2-Benzacridine	−0.016	0	
7-Methyl-3,4-benzacridine	−0.013	0	
5-Methyl-3,4-benzacridine	−0.011	0	
7-Methyl-1,2-benzacridine	−0.003	0	
5,8-Dimethyl-3,4-benzacridine	+0.002	+	0
5,7-Dimethyl-3,4-benzacridine	+0.003	0	
5,9-Dimethyl-3,4-benzacridine	+0.005	0	+
8-Methyl-1,2-benzanthracene	+0.010	+	0
5-Methyl-1,2-benzacridine	+0.011	+++	
6-Methyl-1,2-benzanthracene	+0.013	+	
7-Methyl-1,2-benzanthracene	+0.014	+	+
5-Methyl-1,2-benzanthracene	+0.016	++	++
9-Methyl-1,2-benzanthracene	+0.016	++	+++
5,9-Dimethyl-1,2-benzacridine	+0.021	+++	+++
5,7-Dimethyl-1,2-benzacridine	+0.024	++++	+++
5,8-Dimethyl-1,2-benzacridine	+0.025	++++	+++
5,8-Dimethyl-1,2-benzanthracene	+0.026		0
6,7-Dimethyl-1,2-benzanthracene	+0.027	+	
10-Methyl-1,2-benzanthracene	+0.027	+++	++++
5,6-Dimethyl-1,2-benzanthracene	+0.029	+++	
5,9-Dimethyl-1,2-benzanthracene	+0.032		++++
5,7,9-Trimethyl-1,2-benzacridine	+0.034	+++	++
8,10-Dimethyl-1,2-benzanthracene	+0.037		+++
5,10-Dimethyl-1,2-benzanthracene	+0.043		++++
9,10-Dimethyl-1,2-benzanthracene	+0.043	++++	+++
5,7,9-Trimethyl-3,4-benzacridine	+0.046	+++	0
5,6,7,9-Tetramethyl-1,2-benzacridine	+0.050	+++	+
6,9,10-Trimethyl-1,2-benzanthracene	+0.056	++++	++
5,9,10-Trimethyl-1,2-benzanthracene	+0.059	++++	+++
5,6,9,10-Tetramethyl-1,2-benzanthracene	+0.072	+++	+

[a] Badger (1948).

benz-ring, and at the K position, have been (arbitrarily) excluded. The correlation in this limited series of compounds is seen to be very satisfactory; but it must be emphasized that the exceptions to the original theory remain in this modified theory.

Attempts have also been made to study the electronic configurations of carcinogenic hydrocarbons by the method of molecular orbitals (Coulson, 1952; Greenwood, 1951). The molecular diagrams for most of the tricyclic, tetracyclic, and pentacyclic ring systems have been cal-

TABLE VI
Excess Charge on the K Region (M.O. Method) and Carcinogenic Activity

Compound	Excess Charge on K Region[a]	Carcinogenic Activity[b]	
		Skin	Subcutaneous Tissue
7-Methyl-3,4-benzacridine	−0.0393	0	
3,4-Benzacridine	−0.0387	0	
5,7-Dimethyl-3,4-benzacridine	−0.0366	0	
5-Methyl-3,4-benzacridine	−0.0360	0	
5,7,9-Trimethyl-3,4-benzacridine	−0.0335	+++	0
5,9-Dimethyl-3,4-benzacridine	−0.0329	0	+
5,8-Dimethyl-3,4-benzacridine	−0.0233	+	0
1,2-Benzacridine	−0.0027	0	
1,2-Benzanthracene	0	0	+
7-Methyl-1,2-benzacridine	0.0004	0	
9-Methyl-1,2-benzanthracene	0.0027	++	+++
6-Methyl-1,2-benzanthracene	0.0031	+	
5,6-Dimethyl-1,2-benzanthracene	0.0122	+++	
5,9-Dimethyl-1,2-benzacridine	0.0354	+++	+++
5-Methyl-1,2-benzacridine	0.0360	+++	
5,7,9-Trimethyl-1,2-benzacridine	0.0385	+++	++
10-Methyl-1,2-benzanthracene	0.0387	+++	++++
5,7-Dimethyl-1,2-benzacridine	0.0391	++++	+++
9,10-Dimethyl-1,2-benzanthracene	0.0414	++++	+++
5,8-Dimethyl-1,2-benzacridine	0.0487	++++	+++
5,6,9,10-Tetramethyl-1,2-benzanthracene	0.0536	+++	+

[a] Greenwood (1951).
[b] Badger (1948).

culated, and the potentially carcinogenic ring systems have all been shown to possess a K region having a particularly high bond order. On the other hand, no simple correlation between carcinogenic activity and bond order is possible, for several noncarcinogenic ring systems (e.g., pentaphene) also have K regions of high bond order. It must be emphasized that the bond order as calculated by this method is not directly

related to electron density, but in many ways it is a much more satisfactory index of the "character" of a bond.

Methyl groups, in general, *decrease* the bond order of the 3,4 bond in 1,2-benzanthracene (Greenwood, 1951), and indeed it is calculated that *all* substituents behave similarly. No correlation is therefore possible between carcinogenic activity and the bond orders of the K region in any series of substituted benzanthracenes. On the other hand, methyl substituents act as slight electron donors, and it is therefore possible to calculate the increased charge on the K region. Similarly, it is possible to calculate the effect of an annular nitrogen atom. This has been done for a number of methylbenzanthracenes and methylbenzacridines (Greenwood, 1951). Examination of Table VI shows that there is a very satisfactory relationship between carcinogenic activity and the excess charge on the K region; and the results are in general agreement with those obtained by the valence-bond treatment. Here again, however, all compounds having substituents in the benz-ring, or at the K position, have been (arbitrarily) excluded, and the exceptions to the original Pullman theory remain unexplained. No simple theory relating the charge on the K region to carcinogenic activity can account for the fact that both 10-cyano-, and 10-methyl-1,2-benzanthracenes are active carcinogens, while the parent compound is inactive or almost so.

11. Reactions with Osmium Tetroxide

The reactions of carcinogenic hydrocarbons have been investigated in some detail and there have been many attempts to associate carcinogenic activity with chemical reactivity. For example, Fieser and Campbell (1938) found that many of the most potent carcinogenic hydrocarbons, including 3,4-benzpyrene and 20-methylcholanthrene, couple with diazonium compounds very readily to form azo dyes; but several other carcinogenic hydrocarbons did not react and a few noncarcinogenic hydrocarbons reacted without difficulty. Many carcinogenic hydrocarbons also react with lead tetraacetate; but here again some active compounds were found to be inert toward this reagent, and some inactive or very feebly active compounds were found to be rapidly attacked (Fieser and Hershberg, 1938). Many other reagents have also been investigated; but in every case reaction occurred at the most reactive *center* in the molecule and only limited correlations with carcinogenic activity could be found.

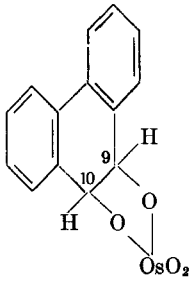
However, there are three reagents which appear to function as "double bond reagents": diazoacetic ester, ozone, and osmium tetroxide (Badger, 1951). These reagents attack the most reactive *bond* in an aromatic molecule and do not attack the most reactive *centers* (unless

these happen to be situated at the extremities of the most reactive bond). In the tricyclic and tetracyclic aromatic hydrocarbons it has been found that these reagents attack the K position (Badger, 1951).

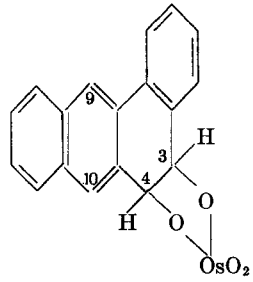
Diazoacetic ester adds to the most reactive bond in an aromatic ring system with elimination of nitrogen. Phenanthrene is attacked at the 9,10 bond, pyrene at the 1,2 bond, and 1,2-benzanthracene at the 3,4 bond (Badger, Cook, and Gibb, 1951).

Ozone also reacts by addition to the most reactive bond. Unfortunately, only a few ring systems have been studied, but it is known that ozone attacks first the 1,2 bond of naphthalene, and it is also known to attack the 1,2 bond in pyrene.

The action of osmium tetroxide has been extensively studied, and in every case it has been found that the reagent adds to the K position (Cook and Schoental, 1948). With phenanthrene, osmium tetroxide adds to the 9,10 bond to give the complex (LXIX). With 1,2-benzanthracene and its derivatives it adds exclusively to the 3,4 bond to give the complex (LXX), in spite of the fact that the 9,10 positions are attacked by most other reagents. Similarly, osmium tetroxide adds to the 1,2 bond of chrysene (XXXIII), to the 1,2-bond of pyrene (XXXV), and to the 6,7-bond of 3,4-benzpyrene (III).



(LXIX)



(LXX)

These results indicated that it might be possible to examine the validity of the various quantum mechanical calculations by measuring the relative rates of addition of osmium tetroxide to the K region of carcinogenic and related noncarcinogenic hydrocarbons. Other things being equal the theoretical calculations would indicate that the carcinogens should be attacked more rapidly than related noncarcinogenic molecules. A method for the determination of the rate of reaction with osmium tetroxide was devised (Badger and Reed, 1948), and numerous carcinogenic and noncarcinogenic hydrocarbons have now been examined (Badger, 1949, 1950; Badger and Lynn, 1950).

Among the unsubstituted ring systems it has been found that there

is a very satisfactory correlation between the relative reactivity and the bond order as calculated by the method of molecular orbitals (Table VII).

TABLE VII
Relative Reactivity and Bond Order

Compound	Relative Reactivity to OsO_4[a]	Bond Order at K Region (M.O. Method)	Total Charge on K Region[b] (V.B. Method)
3,4-Benzpyrene	2.0	1.787[c]	—
1,2,5,6-Dibenzanthracene	1.3	1.778[d]	—
1,2-Benzanthracene	1.0	1.783[e]	1.283[e]
Pyrene	0.66	1.777[e]	—
Phenanthrene	0.1	1.775[f]	1.291[e]
1,2,5,6-Dibenzphenanthrene	slow	1.764[c]	—
Chrysene	slow	1.754[e]	1.272[e]

[a] Badger (1949, 1953); Badger and Reed (1948).
[b] A. Pullman (1947).
[c] Estimated from the bond order-bond localization energy relationship.
[d] Baldock *et al.* (1949).
[e] Berthier *et al.* (1948).
[f] Coulson and Longuet-Higgins (1947).

Relatively few calculations by the valence-bond method for these compounds are available, but it does seem that the correlation between relative reactivity and the total charge on the K region as calculated by this method is less satisfactory.

Substituents have been shown to have a profound influence on the rate of addition of osmium tetroxide to the K position. Methyl groups and other alkyl substituents were found to increase the rate of addition, the magnitude of the effect being determined by the position of substitution. In the alkyl-1,2-benzanthracenes it was at a maximum with the groups in the *meso* positions. 9-Methyl-, 10-methyl-, and 9,10-dimethyl-1,2-benzanthracenes were found to react much more rapidly than the parent hydrocarbon; and 9,10-diethyl-1,2-benzanthracene reacted slightly less rapidly than 9,10-dimethyl-1,2-benzanthracene. Methyl substitution at other positions in the molecule had a smaller effect, 6-methyl- and 5,6-dimethyl-1,2-benzanthracenes being only slightly more reactive than the unsubstituted hydrocarbon. 2′,7-Dimethyl-1,2-benzanthracene was also found to react more rapidly than 1,2-benzanthracene.

Cholanthrene and methylcholanthrene can also be considered as alkyl substituted benzanthracenes and these compounds also reacted more rapidly than 1,2-benzanthracene, as did acenaphthanthracene. The latter observation is interesting as this compound (4′,3-ace-1,2-benzanthracene)

is substituted at the K position, but no pronounced steric hindrance seems to be involved in the addition. 3-Methyl-1,2-benzanthracene also reacted somewhat more rapidly than 1,2-benzanthracene itself.

It is noteworthy that 5,7-dimethyl-1,2-benzacridine reacted only slightly less rapidly than the closely related 10-methyl-1,2-benzanthracene (the 10 position in benzanthracene is equivalent to the 5 position in 1,2-benzacridine).

The carcinogenic 1,2-dimethylchrysene also reacted more rapidly than chrysene, so that the activating influence of methyl groups is quite general.

It is interesting to compare these relative reactivities with the total charge of π electrons at the K region, or with the excess charge at this K region, as calculated by the V.B. method or by the method of molecular orbitals. This is done in Table VIII, and it must be admitted that the correlation is as good as could be expected.

TABLE VIII
Relative Reactivity and Excess Charge on the K Region

Compound	Relative Reactivity to OsO_4[a]	Excess Charge on K Region (V.B. Method)	Excess Charge on K Region (M.O. Method)
1,2-Benzanthracene	1.00	0	0
3-Methyl-1,2-benzanthracene	1.04	—	−0.164
6-Methyl-1,2-benzanthracene	1.33	0.013	+0.003
5,6-Dimethyl-1,2-benzanthracene	1.33	0.029	+0.012
5,7-Dimethyl-1,2-benzacridine	1.67	0.024	+0.039
2',7-Dimethyl-1,2-benzanthracene	1.73	0.035	+0.048
10-Methyl-1,2-benzanthracene	1.90	0.027	+0.039
9-Methyl-1,2-benzanthracene	2.0	0.016	+0.003
Cholanthrene	2.1	(0.036)	—
20-Methylcholanthrene	2.3	(0.047)	—
9,10-Dimethyl-1,2-benzanthracene	5.6	0.043	+0.041
5,6,9,10-Tetramethyl-1,2-benzanthracene	6.0	0.072	+0.054

[a] Badger (1949, 1950).

The effects of some other substituents on the rate of addition have also been investigated. The acetoxymethyl group was found to increase the rate, but to a lesser degree than a methyl group. 10-Acetoxymethyl-1,2-benzanthracene formed an addition complex with osmium tetroxide more rapidly than 1,2-benzanthracene, but less rapidly than 10-methyl-1,2-benzanthracene. *meso*-Acetoxy and *meso*-bromo groups were also found to retard the reaction, and *meso*-methoxy groups had little or no effect on the rate. *meso*-Phenyl groups, however, increased the rate of addition. A 10-cyano group was found to retard the addition.

Only a few of the compounds examined have been tested for carcinogenic activity, but those which have are included in Table IX. As the

TABLE IX
Relative Reactivity and Carcinogenic Activity

Compound	Relative Reactivity to OsO_4[a]	Carcinogenic Activity[b]
9,10-Dimethyl-1,2-benzanthracene	5.6	++++
9,10-Diethyl-1,2-benzanthracene	4.4	+++
9-Methyl-1,2-benzanthracene	2.0	+++
10-Methyl-1,2-benzanthracene	1.90	++++
9,10-Diphenyl-1,2-benzanthracene	1.46	0
10-Acetoxymethyl-1,2-benzanthracene	1.27	+
1,2-Benzanthracene	1.00	±
10-Cyano-9-methyl-1,2-benzanthracene	0.83	++++
10-Bromo-1,2-benzanthracene	0.56	0
9,10-Diacetoxy-1,2-benzanthracene	0.44	0
10-Cyano-1,2-benzanthracene	Slow	+

[a] Badger (1949, 1950).
[b] Badger (1948).

osmium tetroxide reaction can be used as a measure of the excess charge on the K position, the available results do clearly indicate that both electron-attracting and electron-releasing groups convert 1,2-benzanthracene into a cancer-producing substance.

In concluding this section it is of interest that Kooyman and Heringa (1952) have recently suggested that reactivity toward free radical reagents may also be involved in the mechanism of carcinogenesis. Further experimental data on this hypothesis will be awaited with interest.

12. Conclusions

Among the methylbenzanthracenes and methylbenzacridines there is a very satisfactory correlation between the carcinogenic activity and the charge on the K region. This correlation is found whether the charge is calculated by the valence-bond method or by the method of molecular orbitals, and although there are some relatively minor discrepancies, the major conclusions of the quantum mechanical calculations have been confirmed by the study of the rates of addition of osmium tetroxide to many derivatives. It is difficult therefore to escape the conclusion that the correlation is significant.

On the other hand, even among substituted benzanthracenes there are many exceptions to the theory, some of which have already been mentioned. Some of these exceptions disappear if certain assumptions

are made. For example, if it is assumed that an unsubstituted benz-ring is necessary before complete contact can be made between the hydrocarbon and cellular component, then the inactivity of all the benzanthracenes substituted in this ring is perfectly reasonable; but the fact remains that similarly substituted benzpyrenes are carcinogenic.

The carcinogenic activity of 9,10-dimethylanthracene is reasonable enough if it is assumed that the requirement is not a phenanthrene type bond, but simply an *activated* bond. In this connection it is interesting that Pullman and Pullman (1948) estimate the charge on the 1,2 bond in 9,10-dimethylanthracene to be 1.303*e*, whereas 1,4-dimethylanthracene has an unsubstituted 5,6 bond with a charge of only 1.284*e*.

The carcinogenic activity of 10-cyano-1,2-benzanthracene, of 10-chloro-1,2-benzanthracene, and of 10-formyl-1,2-benzanthracene is more difficult to explain, unless it is assumed that the substituent itself aids the formation of the complex between the carcinogen and a cellular component. As a matter of fact this does not seem to be altogether unreasonable. The three substituents mentioned would all be expected to form strong hydrogen bonds with the peptide linkages of proteins (or similar receptors), and it seems not unlikely that such bonds may be involved in suitable cases.

The lack of carcinogenic activity in compounds such as 9,10-diphenyl-1,2-benzanthracene can be explained by the assumption that a planar molecule is necessary for complex formation and, as is well known, the phenyl groups in this molecule are disposed at right angles to the benzanthracene ring system. Other large substituents (acetoxy, methoxy) must also be disposed at an angle to the benzanthracene ring system and would hinder complex formation. Furthermore, as has already been mentioned, the larger alkyl substituents also decrease carcinogenic activity, probably by steric hindrance to complex formation.

In the present state of knowledge therefore, it is reasonable to conclude that the carcinogen forms a complex with some cellular component. Complex formation only occurs with molecules of suitable molecular complexity, and is prevented by large substituents, and by the presence of substituents in certain parts of the molecule; and in general it does not occur if the molecule is buckled by hydrogenation. On the other hand, complex formation is greatly facilitated by the presence of an activated K region; but the necessary degree of activation may vary considerably, depending on the molecular complexity. And finally, it seems possible that complex formation may be assisted by the presence of substituent groups which can form hydrogen bonds with the receptor molecule.

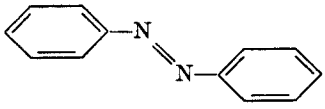
The relationship between chemical constitution and carcinogenic activity in this series is certainly becoming clearer, and it is to be hoped

that future work will enable the mechanism of the process of carcinogenesis to be elucidated.

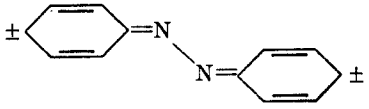
IV. Azo Compounds

1. The Structure of Aromatic Azo Compounds

Azobenzene is the simplest aromatic azo compound. It is a stable bright red highly crystalline substance in which two benzene rings are linked through two doubly bound nitrogen atoms, as in (LXXI).* The double bond is conjugated with the aromatic rings, and structures such as (LXXII) must also contribute to the resonance hybrid. The central N=N bond cannot therefore be a pure double bond, but must have a fractional double bond character, and this is confirmed by analysis of the x-ray diffraction pattern. The percentage double bond character clearly depends on the contribution of the ionic structures such as (LXXII), and in related aromatic azo compounds (such as 1,1′-azonaphthalene and 2,2′-azonaphthalene) this depends on the conjugating abilities of the positions to which the azo group is linked: the greater the conjugating ability, the smaller the double bond character of the central N=N bond (Badger and Lewis, 1951).



(LXXI)



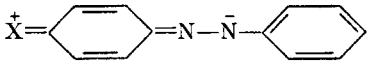
(LXXII)

Each nitrogen atom has a "lone pair" of electrons, and in the aromatic azo compounds these are also shared to some extent with the ring system and are thus relatively unavailable for salt formation.

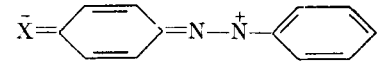
In unsubstituted azo compounds the electron density around the N=N bond therefore depends on the degree of conjugation with the particular aromatic systems involved. In substituted azo compounds, however, the substituent may either increase or decrease the charge on the two nitrogen atoms and may profoundly affect the basicity and the reactivity of the compound. For example, if there is an electron-donating substituent in the 4 position, then structures such as (LXXIII) must contribute to the hybrid; this is equivalent to saying that such a substituent increases the electron density around the nitrogen further removed from it. On the other hand, if there is an electron-attracting

* *cis*-Azobenzene is also known. All the carcinogenic azo compounds are probably of *trans* configuration, however, and *cis* compounds will not therefore be considered here.

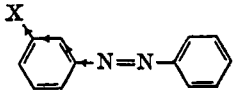
substituent in the 4 position, then structures such as (LXXIV) must contribute to the hybrid; and this is equivalent to saying that the substituent decreases the electron density around the nitrogen further from it.



(LXXIII)



(LXXIV)

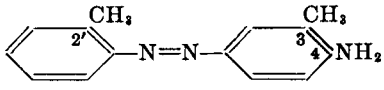


(LXXV)

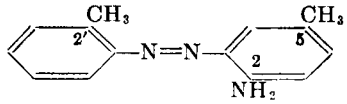
Substituents in the 3 or 3′ position are not conjugated with the azo linkage, but may still affect the electron density around the nitrogen atoms by virtue of their inductive effects. In these circumstances (LXXV) it is the nitrogen atom nearer to the substituent which probably suffers the greater increase or decrease in charge.

2. General Survey of Carcinogenic Azo Compounds

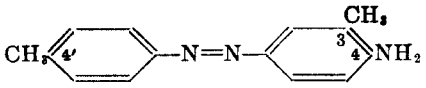
Historically, the most important carcinogenic azo compound is 4-amino-2′,3-dimethylazobenzene, or *o*-aminoazotoluene (LXXVI). Several isomeric compounds have been tested, however, and it seems that a *p*-amino group is not essential for activity (Crabtree, 1949). 2-Amino-2′,5-dimethylazobenzene (LXXVII), for example, also produces liver tumors in both rats and mice. Both of these compounds have a methyl group in the 2′ position, and this does seem to be of some importance, for 4-amino-3,4′-dimethylazobenzene (LXXVIII) is inactive in rats and only slightly active in mice; and 2-amino-4′,5-dimethylazobenzene (LXXIX) appears to be inactive in both species.



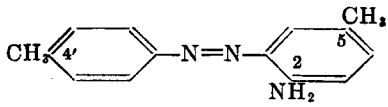
(LXXVI)



(LXXVII)



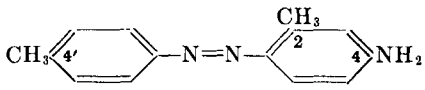
(LXXVIII)



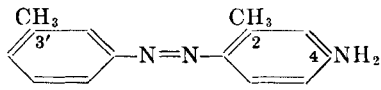
(LXXIX)

It is of some interest that the two additional isomers tested by Crabtree (1949) are also inactive in rats, but active in mice. 4-Amino-2,4′-dimethylazobenzene (LXXX) is relatively active and 4-amino-2,3′-

dimethylazobenzene (LXXXI) rather less so, so that both methyl groups have some influence on the carcinogenic potency.

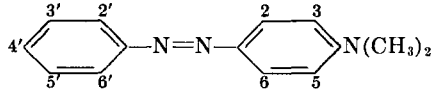


(LXXX)



(LXXXI)

4-Dimethylaminoazobenzene (LXXXII) is also isomeric with the aminoazotoluenes, and most of the known carcinogenic azo compounds are derivatives of this substance, or are closely related to it (Badger and Lewis, 1952). 4-Methylaminoazobenzene appears to be equally active; but 4-aminoazobenzene is much less active and gives liver tumors only when tested under rather special conditions. The higher alkyl derivatives, such as 4-diethylaminoazobenzene and 4-di-*n*-propylaminoazobenzene, are all inactive.



(LXXXII)

Many substituted 4-dimethylaminoazobenzenes have been tested and the results are summarized in Table X. It is seen that both the nature of the substituent and its position have a profound effect on the carcinogenic activity. It is noteworthy that all the hydroxy derivatives which have been tested are inactive, and the various trifluoromethyl derivatives have likewise all failed to produce tumors. The effect of a methyl substituent depends very much on its position. If it is in the 2 or the 3 position the compound is inactive or only very feebly active; if it is in the 2′ or 4′ position the compound is slightly active; but if it is in the 3′ position the compound is more active than the parent substance. Several other substituents are similar and have a variable effect on the carcinogenic activity, and in general the 3′-substituted derivatives are much more potent than the 2′- or 4′-substituted compounds. The fluoro group appears to be exceptional, for all the fluoro-substituted 4-dimethylaminoazobenzenes that have been tested are potent liver carcinogens, and several are more active than the parent compound.

The effect of substitution is therefore not a simple one, and the observed result in any particular case may well be the resultant of several factors.

3. The Azonaphthalenes

2,2′-Azonaphthalene (LXXXIII) has been shown to produce liver tumors in a large proportion of the mice to which it was administered either by mouth, by subcutaneous injection, or by application to the

TABLE X
Substituted 4-Dimethylaminoazobenzenes[a]

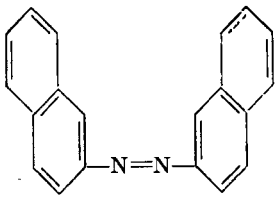
Compound	Carcinogenic Activity in Rats
4-Dimethylaminoazobenzene	++
2-Methyl-	0
3-Methyl-	±
2-Hydroxy-	0
2-Fluoro-	+++
2'-Hydroxy-	0
2'-Methyl-	+
2'-Nitro-	+
2'-Chloro-	+
2'-Fluoro-	++
2'-Trifluoromethyl-	0
2'-Carboxy-	+
3'-Hydroxy-	0
3'-Methyl-	+++
3'-Nitro-	++
3'-Chloro-	++
3'-Fluoro-	+++
3'-Trifluoromethyl-	0
3'-Ethoxy-	±
4'-Hydroxy-	0
4'-Methyl-	±
4'-Nitro-	0
4'-Chloro-	+
4'-Fluoro-	+++
4'-Trifluoromethyl-	0

[a] Badger and Lewis (1952).

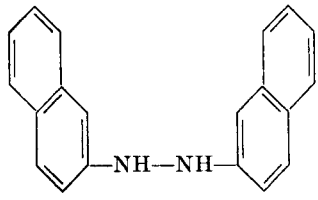
skin. Most of the tumors were cholangiomas, but some hepatomas were also obtained. 1,2'-Azonaphthalene was found to be inactive, but 1,1'-azonaphthalene has produced liver tumors in mice, and 1-benzeneazo-2-naphthol is also active in this respect (Cook, Hewett, Kennaway, and Kennaway, 1940; Kirby and Peacock, 1949). The latter observation is of some interest as this compound has been used until quite recently as a food coloring matter. On the other hand, 4-dimethylaminophenylazo-1'-naphthalene and 4-dimethylaminophenylazo-2'-naphthalene were found to be inactive in rats.

There seems to be some justification for a separate consideration of the azonaphthalenes. Unlike the aminoazotoluenes, 4-dimethylaminoazobenzene, and related compounds, 2,2′-azonaphthalene has no amino group. Moreover, although this compound is a liver carcinogen in mice, it appears to be inactive, or only very slightly active, in rats, and it is not unlikely therefore that there is some fundamental difference (Cook, Hewett, Kennaway, and Kennaway, 1940; Badger, Lewis, and Reid, 1953).

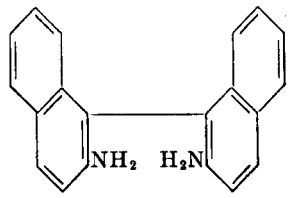
It is possible that such a fundamental difference may be found in the nature of the metabolic products, and in this connection it is interesting that Cook, Hewett, Kennaway, and Kennaway (1940) have suggested that the azonaphthalenes may be transformed in the liver into dibenzcarbazoles. For example, in the case of 2,2′-azonaphthalene (LXXXIII) it was suggested that the true carcinogen, formed in the liver, may be 3,4,5,6-dibenzcarbazole (LXXXVI). This transformation can be brought about without difficulty in the laboratory. Reduction of the azo compound *in vitro* gives the hydrazo derivative (LXXXIV) and this undergoes the benzidine rearrangement in the presence of acids to give 2,2′-diamino-1,1′-dinaphthyl (LXXXV), which on deamination gives 3,4,5,6-dibenzcarbazole (LXXXVI).



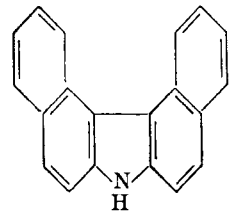
(LXXXIII)



(LXXXIV)



(LXXXV)



(LXXXVI)

The diamine (LXXXV) has been found to be even more active than 2,2′-azonaphthalene in the production of liver tumors in mice, and 3,4,5,6-dibenzcarbazole produces tumors not only in the liver but it is also a potent carcinogen to skin and subcutaneous tissue.

There is no direct experimental evidence that such a transformation

does take place, but it does seem to be a reasonable explanation of the results obtained with the azonaphthalenes. It may well be that rats are unable to effect this transformation and this may explain the inactivity of 2,2′-azonaphthalene in this species. In this connection it may be noted that 3,4,5,6-dibenzcarbazole does produce sarcomas when injected into rats.

Similar transformations have been carried out with the other azonaphthalenes, and here again the experimental results are consistent with the hypothesis of Cook, Hewett, Kennaway, and Kennaway (1940).

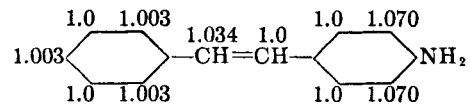
1,1′-Azonaphthalene has only a very slight action on the liver, and 1,2,7,8-dibenzcarbazole, which can be obtained from it, has no action on the liver and is only very slightly active when applied to the skin. 1,2′-Azonaphthalene seems to have no action on the livers of mice and 1,2,5,6-dibenzcarbazole, which can be obtained from it, also appears to be without action on the liver and is only slightly active when applied to the skin.

4. The Influence of the N=N Bond

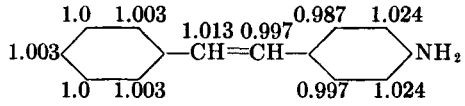
The outstanding feature of the azo compounds is the unsaturated N=N bond with the two "lone pairs," and it is natural to suppose that carcinogenic activity in this series may be associated with this group.

Substituents have a profound effect on the electron density around the two nitrogen atoms, and it has been suggested that the essential requirement for carcinogenic activity is a certain critical charge at this region, called the K′ region (Pullman and Pullman, 1946). This hypothesis is essentially similar to that associating the carcinogenic activity of the polycyclic aromatic hydrocarbons with the charge at the K region.

It has not yet been possible to calculate the electron densities at the K′ region for any carcinogenic azo compounds; but the molecular diagram for the closely related substance, 4-aminostilbene, has been calculated by the valence bond method (A. Pullman, 1948) and by the method of molecular orbitals (Coulson and Jacobs, 1949). The two methods give quite different numerical values for the charge migrations (LXXXVII, LXXXVIII), but qualitatively the agreement is satisfactory. The two positions *ortho* to the amino group are calculated to have a greatly increased charge, and the electron density around the carbon atom further from the amino group is also increased.



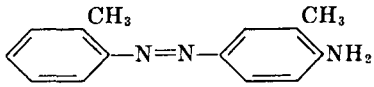
(LXXXVII)
(V.B. method)



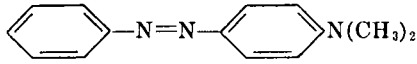
(LXXXVIII)
(M.O. method)

The charge migrations in 4-aminoazobenzene are undoubtedly similar, and although accurate calculations cannot be carried out for substituted azobenzenes the effects of the various substituents can be estimated qualitatively. According to the Pullman hypothesis the azo compounds can be divided into three groups according to the magnitude of the estimated charge on the K′ region (Pullman and Pullman, 1946).

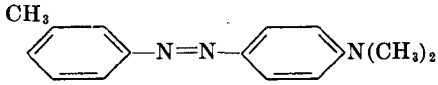
The first group includes the active compounds. These are the substances which are thought to have an electron density at the K′ region between the postulated upper and lower limits. *o*-Aminoazotoluene (LXXXIX), 4-dimethylaminoazobenzene (XC), and 3-methyl-4-dimethylaminoazobenzene (XCI) must be included in this group.



(LXXXIX)

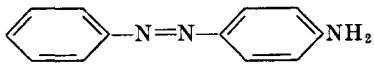


(XC)

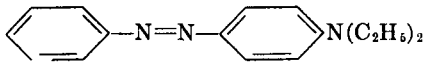


(XCI)

The second group includes all the inactive or feebly active compounds which are thought to have an insufficient charge at the K′ region. In this group Pullman and Pullman (1946) include 4-aminoazobenzene (XCII) and 4-diethylaminoazobenzene (XCIII).

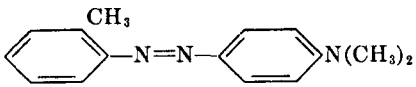


(XCII)

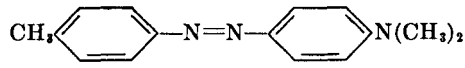


(XCIII)

The third group includes all the inactive or only feebly active compounds which are thought to have a charge at the K′ region which is above the optimum. In this group, Pullman and Pullman (1946) include 2′-methyl-4-dimethylaminoazobenzene (XCIV), 4′-methyl-4-dimethylaminoazobenzene (XCV) and also the 2-methyl derivative.



(XCIV)



(XCV)

This hypothesis has much to commend it, and it does offer a reasonable explanation for the effects of methyl groups and of some other substituents; but the hypothesis is not entirely satisfactory. It is surprising that the effect of a 2′-methyl group on the carcinogenic activity should be almost the same as that of a 2′-nitro group; and the effect of a 4′-methyl group is likewise similar to that of a 4′-nitro group. The effect of a trifluoromethyl group seems reasonable enough as this group is strongly electron attracting; but it is surprising that the 3′-ethoxy derivative is only feebly active; and the high potency of all the fluoro derivatives compared with other halogenated compounds is also somewhat surprising.

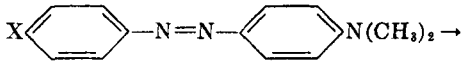
It has been found that when carcinogenic azo compounds are fed to rats, part of the dye becomes very highly bound in the liver, a protein–azo compound complex being formed (Miller and Miller, 1947). The very active compounds give higher levels of bound azo dye in a shorter time than the less active compounds (Miller, Miller, Sapp, and Weber, 1949), and it seems likely that carcinogenic activity is intimately connected with ability to form these complexes in the liver. It may well be, therefore, that factors which assist or hinder the formation of such complexes play an important role in carcinogenesis. Large substituents, for example, might be expected to hinder the formation of such complexes; and electron-attracting substituents might possibly assist complex formation by hydrogen bonding to the receptor molecule.

In any case, the experimental evaluation of the electron densities at the K′ region of a series of carcinogenic and noncarcinogenic azo compounds is clearly an urgent task.

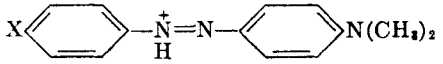
5. Experimental Evaluation of the K′ Region

Relatively little work has so far been carried out on the experimental evaluation of relative electron densities at the K′ region of azo compounds. Nevertheless it should be possible to do this by studying the addition of electrophilic reagents, and two groups of workers have started investigations in this field.

Rogers, Campbell, and Maatman (1951) have examined a number of 4′-substituted 4-dimethylaminoazobenzenes (XCVI) and have found that they all add a proton to one of the nitrogen atoms of the K′ region in dilute acid solution (XCVII). In strong acid solution a second proton is added to the dimethylamino nitrogen atom.



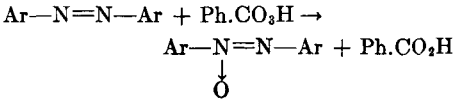
(XCVI)



(XCVII)

The nature of the 4′ substituent was found to have a considerable effect on the ionization constant. Electron-donating substituents were found to promote the addition of the first proton to the K′ region (increase the basicity), and electron-attracting substituents reduced the tendency to add the first proton and decreased the basicity. These results are, of course, in accord with the view that electron-donating substituents increase the charge on the K′ region, and that electron-attracting substituents decrease it. Indeed, the effect of the 4′ substituents on the proton affinity of the azo nitrogen atoms in 4-dimethylaminoazobenzene was found to be in the same order as the net electron affinities of the groups as measured by Hammett's substituent constants, "σ" (Hammett, 1940). As will be seen later this is an important finding.

Another line of approach has been to study the rate of oxidation of a number of substituted azobenzenes with perbenzoic acid (Badger and Lewis, 1951, 1953). Perbenzoic acid is known to be an electrophilic reagent, and it reacts with azo compounds to give the corresponding azoxy derivatives.



In a series of closely related compounds, therefore, the rate of reaction may be taken as a measure of the relative electron density at the K′ region: the greater the electron density at the nitrogen atoms, the greater the reactivity toward electrophilic reagents. Steric hindrance must also be an important factor in some cases, and this generalization would not apply in *ortho*-substituted azo compounds.

A method for carrying out the reaction under standard conditions, in benzene solution, has been worked out and the rates of oxidation of more than twenty substituted azobenzenes have been determined at three temperatures. The rate constants at 25°C. are given in Table XI.

2,2′-Dimethylazobenzene was found to react much slower than the parent compound and this is no doubt due to the steric interference of the *ortho* substituents. In all other cases, however, the rate of reaction clearly depends largely on the electronic character of the substituent. It is found

that there is a smooth curve relationship between the logarithms of the rate constants and the Hammett substituent constants, and these results are therefore complementary to those obtained by Rogers, Campbell, and Maatman (1951).

TABLE XI

Rates of Reaction of Perbenzoic Acid with Substituted Azobenzenes, at 25°C.[a]

Substituent(s)	10^3k_2 (liters g. mole^{-1} min.$^{-1}$)
4,4′-Dimethoxy-	179
4-Methoxy-	58.2
4,4′-Dimethyl-	49.1
4-Methyl-	28.2
3,3′-Dimethyl-	23.9
3-Methyl-	17.1
3-Methoxy-	14.4
Parent compound (azobenzene)	13.9
4-Fluoro-	9.73
4-Chloro-	7.54
4-Bromo-	7.09
3-Carbethoxy-	5.65
4-Carbethoxy-	5.03
3-Bromo-	4.51
4,4′-Dichloro-	4.38
2,2′-Dimethyl-	4.36
3-Chloro-	4.33
4,4′-Dibromo-	4.10
3-Fluoro-	4.06
3-Nitro-	2.05
3,3′-Dichloro-	1.45
4-Nitro-	0.416

[a] Badger and Lewis (1953).

In view of these correlations, it should be possible to compare the charges at the K′ region for a number of substituted 4-dimethylaminoazobenzenes by comparing the Hammett substituent constants. In other words, the greater the positive value of the substituent constant for the substituent in the 3′ or 4′ position, the smaller the charge on the K′ region; and the greater the negative value of the substituent constant for the substituent in the same positions, the greater the charge on the K′ region.

The carcinogenic activities for some 3′- and 4′-substituted 4-dimethylaminoazobenzenes are compared with the substituent constants for the substituents in Table XII. Only a few compounds can be compared in this way, but the available results are consistent with the view that

carcinogenic activity is associated with an optimum charge on the K′ region. In the presence of a strongly deactivating substituent (e.g., 4′-nitro-) the charge on the K′ region may be below the critical limit; and in the presence of an electron-donating substituent (e.g., 4′-methyl-) the charge on the K′ region may approach the critical upper limit; the compounds in both the extreme cases are inactive or only very feebly active. Further compounds must clearly be examined, however, before any supposed correlation can be confirmed.

TABLE XII

Hammett's Substituent Constants and Carcinogenic Activities of Some 4′-Substituted 4-Dimethylaminoazobenzenes

Substituent	Hammett's Substituent Constant[a]	Carcinogenic Activity[b] (Rats)
4′-Nitro-	+0.778; +1.27[c]	0
3′-Nitro-	+0.710	++
3′-Chloro-	+0.373	++
3′-Fluoro-	+0.337	+++
4′-Chloro-	+0.227	+
3′-Ethoxy-	+0.15	±
4′-Fluoro-	+0.062	+++
Parent compound	0.000	++
3′-Methyl-	−0.069	+++
3′,5′-Dimethyl-	−0.138	0
4′-Methyl-	−0.170	±

[a] Hammett (1940).
[b] Badger and Lewis (1952).
[c] Hammett gives two values (0.778 and 1.27) for a *p*-nitro group.

Badger and Lewis (1951, 1953) have also studied the rates of oxidation of the three azonaphthalenes and the two phenylazonaphthalenes, with perbenzoic acid (Table XIII). In these compounds the differences in the electron densities at the K′ region are associated with differences in the conjugating abilities of the 1′ and 2′ positions in naphthalene and of the phenyl ring; but steric factors are also involved in reactions with 1-substituted naphthalenes.

Apart from 1,1′-azonaphthalene, the differences in the rates of reaction with perbenzoic acid are relatively small, and although it was at first thought that these differences might be significant in carcinogenesis (Badger and Lewis, 1951), this now seems unlikely. It has not been possible as yet to determine accurately the rate of reaction of perbenzoic acid with 4-dimethylaminoazobenzene, but it is known that it reacts very much more rapidly than any of the substituted azobenzenes included in

Table XI. (It must be emphasized that the compounds included in Table XI are substituted azobenzenes, and not substituted 4-dimethylaminoazobenzenes. The presence of a 4-dimethylamino group in each case would probably increase the rates of reaction several hundred times.) In the circumstances, therefore, it seems that the electron density at the K′ region in 2,2′-azonaphthalene is not at all comparable with that in

TABLE XIII

Rates of Reaction of Perbenzoic Acid with Unsubstituted Azo Compounds at 25°C.[a]

Compound	$10^3 k_2$ (liters g. mole^{-1} min.$^{-1}$)
1,1′-Azonaphthalene	2.46
Azobenzene	13.9
1-Phenylazonaphthalene	14.5
2-Phenylazonaphthalene	14.5
1,2′-Azonaphthalene	17.7
2,2′-Azonaphthalene	17.6

[a] Badger and Lewis (1951, 1953).

4-dimethylaminoazobenzene and the other aminoazobenzenes. Once again therefore the evidence indicates that 2,2′-azonaphthalene may act by a different mechanism from that of dimethylaminoazobenzene, and the suggestion that it may be metabolized to 3,4,5,6-dibenzcarbazole seems to be the most reasonable explanation.

6. Conclusions

Although it has not been possible to calculate the molecular diagrams for the carcinogenic azo compounds, methods are now available for the experimental comparison of the electron densities at the K′ regions of a series of related compounds. The hypothesis that carcinogenic activity in this class is associated with an optimum density of electrons at the K′ region has certainly not been proved, but at least the majority of the results are not inconsistent with such a view. Many more compounds must be submitted to biological test before any firm conclusion can be reached.

Assuming the hypothesis to be true it should be possible to predict the relative carcinogenic activities of unknown azo compounds, and subsequent biological assay would provide an excellent test of the hypothesis. In this connection the association of carcinogenic activity with the substituent constant of the substituent in the 3′ or 4′ position of 4-dimethylaminoazobenzene, would be most valuable. Any derivative of 4-dimethylaminoazobenzene substituted in the 3′ or 4′ position with a substituent having a Hammett constant with a greater positive value than about 0.8

or with a greater negative value than about 0.2, should be inactive or only feebly active. The examination of the 4′-cyano and 4′-methoxy derivatives of 4-dimethylaminoazobenzene would be interesting.

It seems to be established that the carcinogenic azo compounds enter into some form of complex with a cellular receptor, so that factors which influence the ease of formation and stability of such a complex may well be of importance. The electron density of the K′ region may be a controlling factor; but large substituents may offer steric hindrance to the formation of the complex, and electron-attracting substituents may assist complex formation by hydrogen bonding with the receptor molecule.

Evidence is accumulating that 2,2′-azonaphthalene is unlike the aminoazobenzenes and that it may act by a different mechanism. In this connection the benzidene rearrangement hypothesis holds the field, and if this can be confirmed it means that the carcinogenic activity of 2,2′-azonaphthalene is due to its metabolic conversion into a carcinogen of the polycyclic type. It is to be hoped that some experimental evidence in connection with this hypothesis will be forthcoming.

ACKNOWLEDGMENT

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REFERENCES

Badger, G. M. 1948. *Brit. J. Cancer* **2,** 309–350.
Badger, G. M. 1949. *J. Chem. Soc.* 456–463.
Badger, G. M. 1950. *J. Chem. Soc.* 1809–1814.
Badger, G. M. 1951. *Quart. Rev. (London)* **5,** 147–170.
Badger, G. M. 1953. Unpublished.
Badger, G. M., Cook, J. W., and Gibb, A. R. M. 1951. *J. Chem. Soc.* 3456–3459.
Badger, G. M., Cook, J. W., Hewett, C. L., Kennaway, E. L., Kennaway, N. M., and Martin, R. H. 1942. *Proc. Roy. Soc. (London)* **B131,** 170–182.
Badger, G. M., and Lewis, G. E. 1951. *Nature* **167,** 403–404.
Badger, G. M., and Lewis, G. E. 1952. *Brit. J. Cancer* **6,** 270–292.
Badger, G. M., and Lewis, G. E. 1953. *J. Chem. Soc.* 2147–2150; 2151–2155.
Badger, G. M., Lewis, G. E., and Reid, R. T. W. 1953. Unpublished.
Badger, G. M., and Lynn, K. R. 1950. *J. Chem. Soc.* 1726–1729.
Badger, G. M., Pearce, R. S., and Pettit, R. 1952. *J. Chem. Soc.* 1112–1116.
Badger, G. M., and Reed, R. I. 1948. *Nature* **161,** 238.
Baldock, G., Berthier, G., and Pullman, A. 1949. *Compt. rend.* **228,** 931–933.
Barry, G., Cook, J. W., Haslewood, G. A. D., Hewett, C. L., Hieger, I., and Kennaway, E. L. 1935. *Proc. Roy. Soc. (London)* **B117,** 318–351.
Berenblum, I., and Schoental, R. 1947. *Brit. J. Cancer* **1,** 157–165.
Bergmann, F. 1942. *Cancer Research* **2,** 660–663.
Berthier, G., Coulson, C. A., Greenwood, H. H., and Pullman, A. 1948. *Compt. rend.*, **226,** 1906–1908.

Bielschowsky, F. 1946. *Brit. J. Exptl. Pathol.* **27,** 54–61.
Bielschowsky, F. 1947. *Brit. Med. Bull.* **4,** 382–385.
Bielschowsky, F., and Bielschowsky, M. 1952. *Brit. J. Cancer* **6,** 89–92.
Bloch, B., and Dreifuss, W. 1921. *Schweiz. med. Wochschr.* **51,** 1033.
Bonser, G. M. 1943. *J. Pathol. Bacteriol.* **55,** 1–6.
Bonser, G. M., Clayson, D. B., and Jull, J. W. 1951. *Lancet* **II,** 286–288.
Boyland, E. 1952. *Cancer Research* **12,** 77–84.
Boyland, E., and Brues, A. M. 1937. *Proc. Roy. Soc. (London)* **B122,** 429–441.
Boyland, E., and Horning, E. S. 1949. *Brit. J. Cancer* **3,** 118–123.
Brock, N., Druckrey, H., and Hamperl, H. 1938. *Arch. exptl. Pathol. Pharmakol.* **189,** 709–731.
Cook, J. W. 1932. *Proc. Roy. Soc. (London)* **B111,** 485–496.
Cook, J. W. 1939. *Ergeb. Vitamin-u. Hormonforsch.* **2,** 213–258.
Cook, J. W. 1943. *Roy. Inst. Chem. (London) Lecture* 36 pp.
Cook, J. W., Duffy, E., and Schoental, R. 1950. *Brit. J. Cancer* **4,** 405–410.
Cook, J. W., Haslewood, G. A. D., Hewett, C. L., Hieger, I., Kennaway, E. L., and Mayneord, W. V. 1937. *Am. J. Cancer* **29,** 219–259.
Cook, J. W., Hewett, C. L., and Hieger, I. 1933. *J. Chem. Soc.* 395–405.
Cook, J. W., Hewett, C. L., Kennaway, E. L., and Kennaway, N. M. 1940. *Am. J. Cancer* **40,** 62–77.
Cook, J. W., Hieger, I., Kennaway, E. L., and Mayneord, W. V. 1932. *Proc. Roy. Soc. (London)* **B111,** 455–484.
Cook, J. W., and Kennaway, E. L. 1938. *Am. J. Cancer* **33,** 50–97.
Cook, J. W., and Kennaway, E. L. 1940. *Am. J. Cancer* **39,** 381–428, 521–582.
Cook, J. W., and Schoental, R. 1948. *J. Chem. Soc.* 170–173; *Nature* **161,** 237–238.
Coulson, C. A. 1952. *Advances in Cancer Research* **1,** 1–56.
Coulson, C. A., and Jacobs, J. 1949. *J. Chem. Soc.* 1983–1986.
Coulson, C. A., and Longuet-Higgins, H. C. 1947. *Rev. sci.* **85,** 929–937.
Coulson, C. A., and Longuet-Higgins, H. C. 1948. *Proc. Roy. Soc. (London)* **A195,** 188–197.
Crabtree, H. G. 1949. *Brit. J. Cancer* **3,** 387–398.
Daudel, P. 1950. *Compt. rend.* **230,** 99–100.
Daudel, R. 1948. *Bull. Cancer* **35,** 110–124.
Daudel, P., and Daudel, R. 1950. *Biol. méd.* **39,** No. 4, 1–36.
Dodds, E. C., Lawson, W., and Williams, P. C. 1941. *Nature* **148,** 142.
Dunlap, C. E., and Warren, S. 1941. *Cancer Research* **1,** 953–954.
Dutra, F. R., and Largent, E. J. 1950. *Am. J. Pathol.* **26,** 197–209.
Edwards, J. E. 1941. *J. Natl. Cancer Inst.* **2,** 197–199.
Eschenbrenner, A. B., and Miller, E. 1945. *J. Natl. Cancer Inst.* **5,** 251–255.
Falk, H. L., and Steiner, P. E. 1952. *Cancer Research* **12,** 30–39.
Falk, H. L., Steiner, P. E., Goldfein, S., Breslow, A., and Hykes, R. 1951. *Cancer Research* **11,** 318–324.
Fieser, L. F. 1938. *Am. J. Cancer* **34,** 37–124.
Fieser, L. F., and Campbell, W. P. 1938. *J. Am. Chem. Soc.* **60,** 1142–1145.
Fieser, L. F., Fieser, M., Hershberg, E. B., Newman, M. S., Seligman, A. M., and Shear, M. J. 1937. *Am. J. Cancer* **29,** 260–268.
Fieser, L. F., and Hershberg, E. B. 1938. *J. Am. Chem. Soc.* **60,** 1893–1896, 2542–2548.
Fischer, B. 1906. *Münch. med. Wochschr.* **53,** 2041–2047.
Goulden, F., and Tipler, M. M. 1949. *Brit. J. Cancer* **3,** 157–160.
Greenstein, J. P. 1947. Biochemistry of Cancer. Academic Press, New York.

Greenwood, H. H. 1951. *Brit. J. Cancer* **5**, 441–457.
Haddow, A. 1947. *Brit. Med. Bull.* **4**, 331–342.
Haddow, A., Harris, R. J. C., Kon, G. A. R., and Roe, E. M. F. 1948. *Phil. Trans. Roy. Soc. (London)* **241**, 147–195.
Haddow, A., and Kon, G. A. R. 1947. *Brit. Med. Bull.* **4**, 314–325.
Hammett, L. P. 1940. Physical Organic Chemistry. McGraw-Hill Book Co., New York.
Hartwell, J. L. 1951. Survey of Compounds Which Have Been Tested for Carcinogenic Activity. 2nd ed., Public Health Service Publication, Washington.
Heston, W. E. 1950. *J. Natl. Cancer Inst.* **11**, 415–423.
Hewett, C. L. 1940. *J. Chem. Soc.* 293–303.
Hewett, C. L., and Martin, R. H. 1940. *J. Chem. Soc.* 1396–1398.
Hieger, I. 1930. *Biochem. J.* **24**, 505–511.
Hill, W. T., Stanger, D. W., Pizzo, A., Riegel, B., Shubik, P., and Wartman, W. B. 1951. *Cancer Research* **11**, 892–897.
Jones, R. N. 1940. *J. Am. Chem. Soc.* **62**, 148–152.
Kennaway, E. L. 1924. *J. Pathol. Bacteriol.* **27**, 233–238.
Kennaway, E. L. 1925. *Brit. Med. J.* **II**, 1–4.
Kennaway, E. L., Kennaway, N. M., and Warren, F. L. 1942. *Cancer Research* **2**, 157–159.
Kinosita, R. 1937. *Trans. Japan. Pathol. Soc.* **27**, 665–727.
Kirby, A. H. M., and Peacock, P. R. 1949. *Glasgow Med. J.* **30**, 364–372.
Kooyman, E. C., and Heringa, J. W. 1952. *Nature* **170**, 661–662.
Korpássy, B., and Mosonyi, M. 1950. *Brit. J. Cancer* **4**, 411–420.
Lacassagne, A., Buu-Hoï, N. P., Hoan, N., and Rudali, G. 1948. *Compt. rend.* **226**, 1852–1853.
Lacassagne, A., Buu-Hoï, N. P., Lecocq, J., and Rudali, G. 1946. *Bull. du Cancer* **33**, 48–59.
Lacassagne, A., Buu-Hoï, N. P., Lecocq, J., and Rudali, G. 1947. *Bull. du Cancer* **34**, 22–29.
Lacassagne, A., Buu-Hoï, N. P., and Rudali, G. 1945. *Brit. J. Exptl. Pathol.* **26**, 5–12.
Larsen, C. D. 1947. *J. Natl. Cancer Inst.* **8**, 99–101.
Leiter, J., and Shear, M. J. 1942. *J. Natl. Cancer Inst.* **3**, 167–174.
Miller, E. C. 1951. *Cancer Research* **11**, 100–108.
Miller, E. C., and Miller, J. A. 1947. *Cancer Research* **7**, 468–480.
Miller, E. C., Miller, J. A., Sandin, R. B., and Brown, R. K. 1949. *Cancer Research* **9**, 504–509.
Miller, E. C., Miller, J. A., Sapp, R. W., and Weber, G. M. 1949. *Cancer Research* **9**, 336.
Nettleship, A., Henshaw, P. S., and Meyer, H. L. 1943. *J. Natl. Cancer Inst.* **4**, 309–319.
Newman, M. S., and Cathcart, J. A. 1940. *J. Org. Chem.* **5**, 618–622.
Newman, M. S., and Kosak, A. I. 1949. *J. Org. Chem.* **14**, 375–381.
Orchin, M. 1951. *J. Org. Chem.* **16**, 1165–1169.
Pullman, A. 1947. *Ann. chim. (Paris)* **2**, 5–71.
Pullman, A. 1948. *Compt. rend.* **226**, 486–488.
Pullman, A., and Pullman, B. 1946. *Rev. sci.* **84**, 145–158.
Pullman, A., and Pullman, B. 1948. *Acta Unio intern. contra Cancrum* **6**, 57–63.
Pullman, B. 1946. *Compt. rend.* **222**, 1396–1398.
Pullman, B. 1948. *Bull. soc. chim.* **15**, 533–550.

Robinson, R. (Sir) 1946. *Brit. Med. J.* **I,** 943–945.
Rogers, M. T., Campbell, T. W., and Maatman, R. W. 1951. *J. Am. Chem. Soc.* **73,** 5122–5124.
Shear, M. J., Leiter, J., and Perrault, A. 1940. *J. Natl. Cancer Inst.* **1,** 303–336.
Steiner, P. E., and Falk, H. L. 1951. *Cancer Research* **11,** 56–63.
Tilak, B. D. 1951. *Proc. Indiana Acad. Sci.* **33,** 131–141.
Waller, R. E. 1952. *Brit. J. Cancer* **6,** 8–21.
Walsh, A. D. 1948. *Quart. Revs. (London)* **2,** 73–91.
Weil-Malherbe, H. 1946. *Biochem. J.* **40,** 351–363.
White, F. R., and Eschenbrenner, A. B. 1945. *J. Natl. Cancer Inst.* **6,** 19–21.
Wilson, R. H., DeEds, F., and Cox, A. J. 1941. *Cancer Research* **1,** 595–608.
Yoshida, T. 1933. *Trans. Japan. Pathol. Soc.* **23,** 636.
Yoshida, T. 1934. *Trans. Japan. Pathol. Soc.* **24,** 523–530.

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Carcinogenesis and Tumor Pathogenesis

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I. Introduction

That tumors may arise through extrinsic action has been known ever since Pott (1775) first reported on the prevalence of scrotal cancer among chimney-sweeps. The experimental production of tumors in animals is of much more recent origin, dating from the observation of Marie *et al.* (1910) on the development of sarcomas in rats irradiated with x-rays, and of Yamagiwa and Itchikawa (1914) on the carcinogenic action of coal tar on the rabbit's skin. An extensive follow-up of experimental carcinogenesis began around 1920, and the literature of the subject has since grown so rapidly that it can no longer be comprehensively surveyed in a single review article. Even the following attempt to restrict the field to one branch, the contributions of experimental carcinogenesis to tumor pathogenesis, lays no claim to completeness, but aims rather at focusing attention on those aspects which, though still controversial, have added most to our present understanding of the subject.

The scope of this review may best be defined by distinguishing *tumor etiology* (concerned with the carcinogenic agents themselves, their chemical interrelationships, and their physical and chemical properties) from *tumor pathogenesis* (concerned with the neoplastic response of the tissues to their action). Only the latter comes within the range of this review. The metabolism of carcinogens in the body, which may ultimately serve

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as a link between tumor etiology and pathogenesis (Boyland, 1950, 1952), will only be briefly touched upon, as its role in carcinogenesis is still obscure. The role of viruses in carcinogenesis will not be discussed at all.

For earlier reviews, see Woglom (1926), Watson (1932), and Seelig and Cooper (1933), on carcinogenesis with coal tar; Cook *et al.* (1937), Cook and Kennaway (1938, 1940), Fieser (1938, 1940) and Wolf (1952), on carcinogenesis with synthetic compounds; and the tabulated survey of carcinogenic compounds, compiled by Hartwell (1951). For reviews on separate aspects, see Badger (1948), on carcinogenesis with polycyclic aromatic hydrocarbons; Shear (1937), Rusch *et al.* (1945a), Cook (1948), Miller and Miller (1948, 1953), and Badger and Lewis (1952), on carcinogenesis with azo compounds; Lacassagne (1936, 1938a), Loeb (1940), Gardner (1947), Lipschutz (1950), Burrows and Horning (1952), and Gardner (1953), on carcinogenesis with hormones; Rusch (1944), on extrinsic factors influencing carcinogenesis; Berenblum (1944), on irritation and carcinogenesis; Cowdry (1947, 1953) and Carruthers (1950), on chemical changes in skin carcinogenesis; Rous and Kidd (1938), and Rogers and Rous (1951), on virus action and chemical carcinogenesis; as well as the collected review articles by Various Authors (1947a,b, 1949).

Experimental carcinogenesis is also dealt with at some length in textbooks and monographs on cancer by Hueper (1942), Oberling (1944), Lacassagne (1946, 1947), Rondoni (1946), Maisin (1948), Bauer (1949), and others; and in publications on specific aspects of cancer, e.g., on the biochemistry of cancer, by Stern and Willheim (1943) and by Greenstein (1947); on vitamins and cancer, by Burk and Winzler (1944); on gastric cancer, by Barrett (1946); and on the role of genetics in cancer, by Little (1947).

The experimental approach to the somatic cell mutation theory of cancer is discussed by Dunning *et al.* (1936, 1940), Rous and Kidd (1941), Cowdry (1947), Demerec (1948), Strong (1949a), Berenblum and Shubik (1949a), Blum (1950), and others. For a more speculative consideration of the subject, including the relation of carcinogenesis to cellular differentiation, virus action, the plasmagene theory, and other aspects of cancer research indirectly related to experimental carcinogenesis, see reviews by Waddington (1935), Berrill (1943), Haddow (1944), Henshaw (1945), Loeb (1947), Medawar (1947), Spiegelman (1947), Darlington (1948), Holtfreter (1948), Strong (1949b), Lipschutz (1951), Berenblum (1952), and Various Authors (1952).

II. Nature of Response to Carcinogenic Action

The experimental induction of a tumor is a relatively simple procedure, though the ease and speed with which the effect is produced

varies with the species and strain of animal. The method involved, whether by repeated applications to the skin, by one or more injections, by continued feeding, or by a more elaborate procedure, is determined partly by the desired site of action and partly by the kind of carcinogen used. There are, in fact, two patterns of carcinogenic action: one, which takes place at the site of administration of the carcinogen, and the other, which occurs remotely in some specific organs or tissues.

The "local" type of carcinogenic action is well exemplified by irradiation with ultraviolet or x-rays and by the administration of the chemical carcinogens which belong to the class of polycyclic aromatic hydrocarbons.

Ultraviolet rays have very limited penetrating power; their carcinogenic effects are, therefore, restricted to the surface of the body, and confined to the area actually subjected to the rays (Findlay, 1928; Rusch *et al.*, 1941; Blum, 1948). With x-rays, the extent and depth of action varies with the wavelength, soft rays being carcinogenic to the tissues near the surface (Marie *et al.*, 1910; Jonkhoff, 1927), while the harder rays also inducing tumors internally, e.g., in the ovaries, myeloid and lymphoid tissues, and breast (Furth and Furth, 1936; Lorenz *et al.*, 1951), connective tissue and bone (Ross, 1936). That carcinogenesis by irradiation is restricted to the cells actually exposed to the rays, is evident also from apparently "remote" carcinogenesis by intravenous injection of radium (Sabin *et al.*, 1932) or plutonium and uranium fission products (Lisco *et al.*, 1947), the resulting tumors arising in the particular tissue (bone) in which these radioactive substances are trapped. Similarly, radioactive iodine, injected intraperitoneally, produces tumors of the thyroid (Doniach, 1950; Goldberg and Chaikoff, 1952).

The localized pattern of carcinogenic action by polycyclic aromatic hydrocarbons calls for more detailed consideration.

When such a carcinogen, dissolved in an organic solvent, is repeatedly applied to the mouse's skin, multiple papillomas appear in the painted area after an interval of 6 weeks to a year or more. These papillomas usually grow progressively and eventually become malignant, though some remain stationary or even regress. Less commonly, the tumor is malignant from the start. [The occasional appearance of a skin papilloma or carcinoma outside the painted area, probably arises from mechanical spread of the carcinogen through scratching or licking by the animal (Lefèvre, 1945), though this explanation has been questioned (see Law, 1941c).] Basal cell carcinomas (Oberling *et al.*, 1939a) or malignant melanomas (Berenblum, 1949) of the skin are less commonly induced by painting than papillomas and squamous carcinomas.

If instead of being applied to the skin, the carcinogen is injected

subcutaneously, the resulting lesion, appearing after 3–18 months, is a sarcoma, again arising at, or very close to, the site of administration. When injected into parenchymatous organs or specialized tissues, these carcinogens induce tumors of specialized cell types characteristic of the tissues affected, e.g., tumors of smooth or striated muscle (Haagensen and Krehbiel, 1936), uterus (Ilfeld, 1936), prostate (Moore and Melchionna, 1937), bone (Brunschwig and Bissell, 1938), brain, kidney (Seligman and Shear, 1939), breast (Strong and Smith, 1939), thymus (often with accompanying leukemia), testis (Rask-Nielsen, 1948), lung (Rask-Nielsen, 1950a), urinary bladder (Jull, 1951).

When these compounds are given by mouth, tumors tend to develop along the gastrointestinal tract, appearing as squamous carcinoma of the cardiac (squamous) portion of the stomach (Klein and Palmer, 1940; Stewart and Lorenz, 1942) and adenocarcinoma of the small intestine (Lorenz and Stewart, 1940). Adenocarcinoma of the glandular portion of the stomach can be induced by injecting these compounds into the wall of that organ (Stewart and Lorenz, 1942; Hare *et al.*, 1952).

Localized action of polycyclic aromatic hydrocarbons on specific tissues can also be brought about by incorporating the carcinogen in a slice or mince of the particular tissue and injecting the material subcutaneously. Tumors have been successfully induced by this method in mice with embryonic intestinal mucosa, lung, muscle, cartilage (Greene, 1945), skin, and stomach mucosa (Greene, 1945; Rous and Smith, 1945; Smith and Rous, 1945), and also with adult tissues, such as prostate (Horning, 1946) and lung (Horning, 1947). In fowls, only sarcoma of the host tissues develop, according to Vigier and Guerin (1952). The latent period for tumor production by this procedure is remarkably short, compared to other standard methods of local carcinogenesis. Some discrepancies have still to be clarified, as, for instance, Greene's reported induction of glandular carcinoma with stomach mucosa, while Rous and Smith only succeeded in getting squamous carcinoma. The former also claimed that the method was effective irrespective of the species or strain of animal used for donor or recipient, while the latter found evidence of strict genetic specificity.

Summarizing, it would seem that the "locally acting" carcinogens belonging to the class of polycyclic aromatic hydrocarbons are potentially carcinogenic for all tissues. In practice, this operates only in a restricted sense, since a sarcoma often develops from the stroma of the parenchymatous organ into which the carcinogen is injected, before the specialized cells of that organ have time to respond. As the relative efficacy for producing carcinoma and sarcoma, respectively, is not the same for all carcinogenic hydrocarbons (Rask-Nielsen, 1948, 1950a), it may well be

that with a carcinogen possessing a relatively weaker "sarcogenic" than "carcinogenic" potency, tumors of specialized cell types would be more readily elicited than is the case with the three carcinogens (1,2,5,6-dibenzanthracene, 3,4-benzypyrene, and 20-methylcholanthrene) most extensively tested so far.

In contrast to polycyclic aromatic hydrocarbons, other "locally acting" carcinogens are usually effective for one or two tissues only. Some are only carcinogenic to the skin, e.g., arsenic (Leitch and Kennaway, 1922), conc. aqueous HCl or NaOH (Narat, 1925), oleic acid (Twort and Fulton, 1930), and various quinones (Takizawa, 1940b). A new series of aliphatic compounds—various bis-epoxides—were recently found to produce tumors of the skin, by painting, and subcutaneously, by injection (Hendry *et al.*, 1951). Others only produce sarcomas, e.g., tin metal (Larionov, 1930), styryl 430 (Browning *et al.*, 1936), conc. solutions of glucose and other sugars (Takizawa, 1940a), dil. HCl in phthalate buffer (Suntzeff *et al.*, 1940), deoxycholic acid (Badger *et al.*, 1940), polymerized plastics, such as bakelite (Turner, 1941) and cellophane (Oppenheimer *et al.*, 1948), nickel powder (Hueper, 1951), uranium metal (Hueper *et al.*, 1952), and (in rats only) even such apparently innocuous materials as olive oil and lard (Burrows *et al.*, 1936). Zinc chloride is carcinogenic for the testis of the fowl (Michalowsky, 1928), while alcohol is carcinogenic for the mucosa of the mouth and of the rectum in the mouse (Krebs, 1928); chromium and cobalt induce sarcomas when introduced directly into bone (Schinz and Uehlinger, 1942), while beryllium salts (Gardner and Heslington, 1946) and beryllium metal (Barnes, 1950) have a similar predilection for bone, where they are deposited following intravenous injection. *In most cases where local carcinogenesis is restricted to one tissue, the carcinogenic action is weak, the tumor yield being low and the latent period rather long.*

Unlike the "locally acting" carcinogens, which induce their tumors at the sites of administration, "remotely acting" carcinogens induce tumors in certain specific organs or tissues, irrespective of the manner or route of administration, the tissue selectivity varying according to the compound used, though influenced also by the type of species and strain of animal (see below). Chemically, the remotely acting carcinogens constitute a heterogeneous collection, as the following examples will illustrate.

Estrogens, given by mouth or injection, produce, in mice of certain strains, a high incidence of mammary carcinoma in males as well as females (Lacassagne, 1932, 1938a; Gardner, 1947); also tumors of the pituitary (Cramer and Horning, 1936), lymphoid tissue (Lacassagne, 1937), uterine cervix (Suntzeff *et al.*, 1938), and testis (Hooker *et al.*,

1940; Bonser and Robson, 1940). (For the response of other species, see below.)

Intraperitoneal injections of pituitary growth hormones into rats produce peribronchial lymphosarcomas, adrenal medullary tumors, ovarian tumors, and an increased incidence of mammary fibroadenomas (Moon *et al.*, 1950a,b,c).

When *o*-aminoazotoluene (Sasaki and Yoshida, 1935; Andervont *et al.*, 1942), *p*-dimethylaminoazobenzene (Kinosita, 1937), and some related azo compounds (Miller and Miller, 1948; Rumsfeld *et al.*, 1951) are fed or injected, tumors (of both liver cell and bile duct origin) develop in the liver; and with *p*-dimethylaminoazobenzene, tumors of the pancreas may also develop (Hoch-Ligetti, 1949).

Beta-naphthylamine, given subcutaneously or orally, produces tumors of the urinary bladder in dogs (Hueper *et al.*, 1938), rats, and rabbits (Bonser *et al.*, 1951).

In strains of mice subject to spontaneous lung tumors, an increased incidence occurs after feeding or injection of urethane (Nettleship and Henshaw, 1943); and also after intravenous injections of nitrogen mustard (Boyland and Horning, 1949; Heston, 1950) or sulfur mustard (Heston, 1950). Urethane also produces liver tumors in rats (Jaffé, 1947a); and nitrogen mustard may produce fibrosarcomas, lymphosarcomas, and adenocarcinomas, remotely (Griffin *et al.*, 1950).

An even wider range of action is shown by 2-acetylaminofluorene (Wilson *et al.*, 1941, 1947a; Bielschowsky, 1944; Armstrong and Bonser, 1947; Dunning *et al.*, 1947; Foulds, 1947), which produces tumors of the liver, breast, lung, stomach, intestine, uterus, thyroid, urinary bladder, external auditory duct, etc., all remote from the site of administration, but does not display carcinogenic activity locally. In rabbits, tumors appear only in the urinary bladder and ureter (Bonser and Green, 1951).

Other remotely acting carcinogens include carbon tetrachloride (Edwards, 1941), selenium (Nelson *et al.*, 1943), tannic acid (Korpássy and Mosonyi, 1950), and alkaloids of *Senecio jacobaea* (Cook *et al.*, 1950), all of which induce liver tumors; thiourea, which induces tumors of both the thyroid (Purves and Griesbach, 1947) and the liver (Fitzhugh and Nelson, 1948); and benzidine (Spitz *et al.*, 1950), which induces sebaceous gland carcinoma, hepatoma, and adenocarcinoma of the rectum. Various tumors in different animals have been reported following prolonged administration of acetylcholine (Hall and Franks, 1938).

The difference between locally acting and remotely acting carcinogenesis, in tumor location, may be partly explained by the solubility and diffusibility of the respective classes of compounds: a carcinogen which is relatively inactive toward the cells at the site of administration,

yet sufficiently diffusible to reach distant tissue that are readily responsive, will tend to function as a remotely acting carcinogen; conversely, one which is strongly carcinogenic towards the local tissue to which it is administered, yet not sufficiently diffusible, will function as a locally acting carcinogen.

In fact, locally acting carcinogens do sometimes produce tumors in distant organs, and remotely acting carcinogens occasionally produce tumors locally, as would be expected on the basis of such a simple, physicochemical explanation. Thus, in susceptible strains of mice, an increase in lung tumor incidence may result from subcutaneous injections of dibenzanthracene (Andervont, 1937), while applications of carcinogenic hydrocarbons to the mouse's skin may cause an increase in mammary cancer incidence (Maisin and Coolen, 1936; Engelbreth-Holm, 1941; Lefèvre, 1945; Kirschbaum *et al.*, 1946) and the development of lymphomatosis and leukemia (Morton and Mider, 1938; Law, 1941b; Lefèvre, 1945; Rask-Nielsen, 1950b). Conversely, sarcomas have occasionally been observed at the sites of injection of what are essentially remotely acting carcinogens, such as *o*-aminoazotoluene and *p*-dimethylaminoazobenzene (Law, 1941a), nitrogen mustard (Boyland and Horning, 1949), and even with estrone (Gardner *et al.*, 1936); and skin tumors have occasionally appeared at the site of painting with dimethylaminoazobenzene (Kirby, 1948a).

The crucial experiment would be to determine whether a remotely acting carcinogen is most effective when administered *directly to the target organ for which it is carcinogenic from a distance.* This has been attempted with acetylaminofluorene in rats, applied to the external auditory duct, both in powder form (Wilson *et al.*, 1947b) and in solution in acetone (Berenblum and Haren, 1952: unpublished experiments). No tumors developed locally! Another example is that of β-naphthylamine, a remotely acting carcinogen for the urinary bladder, which fails to induce tumors when introduced directly into that organ (Bonser *et al.*, 1952).

The mechanism determining the localization of carcinogenic action is, therefore, obviously more complicated than that based on principles of solubility and diffusibility. Account must be taken of the fact that carcinogens are metabolized in the body (see reviews: Boyland and Weigert, 1947; Young, 1950), and that the chemical transformation involved may possibly play a part in carcinogenesis (Boyland, 1950, 1952). Species and strain distribution of tumors in response to extrinsic action (see below) could hardly be explained by differences in solubility and diffusibility of the inducing agents, but may well be accounted for by genetic differences of the enzymes concerned with the metabolic conversion of these compounds. Unfortunately, not much is known yet about the role of the metab-

olites of carcinogens in the process of carcinogenesis. The fact that the end products of metabolism of carcinogens possess little or no carcinogenic action (Boyland *et al.*, 1941; Berenblum and Schoental, 1943; Heidelberger and Wiest, 1951), does not necessarily preclude the possibility that some intermediates may be involved in the carcinogenic process. In this connection, the fact that the methylated product of the 8-OH metabolite of benzypyrene is highly carcinogenic (Cook and Schoental, 1952), is very suggestive.

Further complication may be visualized from arguments based on indirect evidence. The liver is one of the most important organs for the metabolic conversion of substances foreign to the body (see review: Williams, 1947), and polycyclic aromatic hydrocarbons are also known to be oxidized in that organ (Peacock, 1936; Heidelberger and Wiest, 1951). Yet, the liver is relatively unresponsive to the carcinogenic action of these carcinogens (Oberling *et al.*, 1936; Shear *et al.*, 1940; Esmarch, 1942). On the other hand, in response to remotely-acting carcinogens, the liver appears to be a favorite target organ for carcinogenesis (Sasaki and Yoshida, 1935; Kinosita, 1937; Wilson *et al.*, 1941; etc.).

If the carcinogenic activity of polycyclic aromatic hydrocarbons were brought about by the parent compounds and not by their metabolites, then the facility of the liver to metabolize (and supposedly destroy) them would account for the relative lack of carcinogenic response by that organ. One could postulate further that remotely acting carcinogens function in the body as precursors of true carcinogens, and that the restriction of their carcinogenic action to certain particular tissues (e.g., the liver) may be dependent on the presence there of the necessary enzymes required for their conversion into locally acting types of carcinogens. In support of this is the recent observation (Bonser *et al.*, 1951, 1952) that 2-amino-1-naphthol, a metabolite of the remotely acting carcinogen, β-naphthylamine, is *locally* carcinogenic for the urinary bladder when introduced directly into that organ. Further evidence would be valuable in clarifying this important aspect of the mechanism of action.

Allied to the question of the localization of induced tumors is the problem of dose-response relationships of carcinogens acting on different tissues.

Useful data are available regarding optimal and minimal requirements for local carcinogenesis by subcutaneous injection of polycyclic aromatic hydrocarbons (Leiter and Shear, 1943; Bryan and Shimkin, 1943; Shimkin and Wyman, 1947). For 100% tumor yield in C3H mice, the requirements for a single injection were found to be 0.5 mg. for 3,4-benzpyrene, 0.125 mg. for 20-methylcholanthrene, and 0.062 mg. for 1,2,5,6-dibenzanthracene, all tested in solution in trycaprylin (Bryan and Shimkin, 1943);

while the lowest effective doses, in tricaprylin, were 0.00195 mg. for benzpyrene (yielding tumors in 2.87% of injected animals), 0.0078 mg. for methylcholanthrene (yielding tumors in 19%) and 0.00195 mg. for dibenzanthracene (yielding tumors in 3.2%). When tested in the form of solid pellets in cholesterol, implanted subcutaneously, the doses for optimal carcinogenesis were higher than with tricaprylin as medium (Shimkin and Wyman, 1947), though the minimal dose requirements remained low, especially for dibenzanthracene, of which 0.002 mg. was effective (and even as low as 0.0004 mg. in one reported case by Shear, 1936).

For skin tumor production, where the carcinogen is applied as a 0.3–0.6% solution in benzene, the amount administered per application is, at most, 0.1 mg. (Cramer and Stowell, 1943). Though skin tumors may develop following a single large dose of carcinogen (Mider and Morton, 1939; Law, 1941c; Cramer and Stowell, 1943), the usual practice for optimal effect, with the majority of skin carcinogens, is to continue the painting at weekly or half-weekly intervals for 20 weeks or more, representing a total dose of at least 3 mg. per mouse.

Accepting 0.05 mg. as the *minimal* dose, and 3.0 mg. as the *optimal* dose, for skin carcinogenesis, and 0.001 mg. and 0.06 mg., respectively, for subcutaneous carcinogenesis, the difference in requirements would appear to be 50 times greater for skin than for subcutaneous carcinogenesis.

This quantitative difference is, however, more apparent than real, since polycyclic aromatic hydrocarbons persist subcutaneously for many weeks or months (Berenblum and Schoental, 1942; Heidelberger and Weiss, 1951), whereas they disappear rapidly from the surface of the skin (Hieger, 1936; Ahlström and Berg, 1947; Heidelberger and Weiss, 1951). In both cases, there is a gradual diffusion of minute amounts of carcinogen into the living cells (Graffi, 1939; Ahlström and Berg, 1947; Setälä, 1949a), but whereas subcutaneously, the bulk remains *in situ* unabsorbed, and can thus continue to act for a long time, in the case of the skin, the greater part is shed from the surface in a matter of days. This would probably suffice to account for the observed fifty-fold difference in dose requirement in the two cases.

With remotely acting carcinogens, the situation is more complex. Lung tumors have been produced in mice by intravenous injection of as little as 0.0015 mg. of dibenzanthracene (Andervont and Lorenz, 1937); but since the carcinogen tends to be filtered out of the blood stream into the lung capillaries (Shimkin and Lorenz, 1942), the resulting tumor formation is more of the nature of locally acting than remotely acting. (Yet, lung tumors also arise following subcutaneous injection of dibenzanthracene (Andervont, 1937a), from which gross particles, capable of being trapped

in the lung, are not likely to be found in the blood stream.) For lung tumor production with nitrogen mustard, the intravenous dose is about 0.1 mg. (4 injections of 0.001 mg. per gram of body weight) in mice (Heston, 1950); and since no more than a small fraction of this is likely to be taken up by the lung tissue, the effective dose, in this case, must also be very small. With estrone, the minimal dose for mammary tumor production is about 1 mg. subcutaneously (Lacassagne, 1938b; Shimkin and Grady, 1940), and about the same for stilbestrol (Shimkin and Grady, 1940). The lowest dose to be effective, by the intraperitoneal route, for tumor production of the lung with urethane is about 20 mg. in aqueous solution (Henshaw and Meyer, 1944).

Larger amounts are needed for some of the other remotely acting carcinogens: e.g., with *o*-aminoazotoluene, 10–100 mg. subcutaneously, in divided doses (Law, 1941a; Andervont *et al.*, 1942), and still larger amounts when given by mouth (Sasaki and Yoshida, 1935); with *p*-dimethyl aminoazobenzene, almost as much (Kinosita, 1937); and with acetylaminofluorene, about 250 mg. by mouth, in divided doses (Wilson *et al.*, 1947a).

So wide a range in minimal requirements, among the remotely acting carcinogens, representing more than a thousand-fold difference between the lowest and highest figures, suggests, at first sight, that different mechanisms might be involved. Yet, simpler explanations are possible, namely, that those carcinogens which act only in large doses, are compounds which are rapidly detoxicated, or rapidly eliminated, so that in order to maintain an adequate *effective* concentration in the tissues, excessive amount must be administered. (Indeed, the water-soluble urethane is rapidly excreted, while the fat-soluble azo compounds are probably rapidly detoxicated.) Another interesting possibility is that those substances that only act in high dose ranges are, in fact, only precursors of carcinogens, which have to be converted in the body into true carcinogens—a suggestion already considered above on different grounds.

This section may be concluded with a brief consideration of methods of assessing carcinogenic activity.

Though the process of carcinogenesis is now known to be made up of separate stages involving independent mechanisms (see below), the ultimate evolution of the tumor is manifestly an all-or-none phenomenon. The quantitative evaluation of the overall process cannot, therefore, be defined in terms of *intensity* of response, but must depend on *the readiness with which the tumor is induced.* This may be expressed quantitatively either as the "average latent period" (i.e., as the time taken to bring about the effect in a given proportion of animals) or as the "percentage tumor yield" (i.e., as the proportion of animals which respond).

Though these two values are not alternative measures of carcinogenic action, but represent two different aspects of the overall process (see below), nevertheless, for practical purposes, the choice is usually determined by the nature and type of investigation. Where the percentage tumor yield is likely to approach 100% in both the experimental and control groups (e.g., in most studies of locally acting carcinogenesis), one usually uses the average latent period as index; though for very accurate comparisons, the percentage tumor yield *determined at different dose levels* is more reliable (Bryan and Shimkin, 1943). Where the resulting tumors are not recognizable till after the animals are sacrificed (e.g., in most studies with remotely acting carcinogens), the percentage tumor yield is usually used as index. The average latent period is, on the whole, the more flexible measure, and is specially applicable for overall comparisons of varied data published by different authors (see grading system by Berenblum, 1945); though a comprehensive "carcinogenic index," incorporating both methods of assay, has been applied by various authors (Iball, 1939; Badger, 1948).

III. Genetic Factors Influencing Carcinogenesis

Since the early studies of skin carcinogenesis with tar (see reviews: Woglom, 1926; Watson, 1932; Seelig and Cooper, 1933), it has been evident that neoplastic response varies with the species of animal used, skin tumors developing readily in rabbits and mice, but not in rats and guinea-pigs (Itchikawa and Baum, 1924). It was later shown that the difference was only a relative one, and that provided the sample of tar was potent enough and the treatment continued long enough, skin tumors could be induced also in the rat (Watson, 1935), and even in such "refractory" animals as the fowl (Choldin, 1927), the monkey (Bonne *et al.*, 1930), and the dog (Passey, 1938), though not, apparently, in the guinea-pig. Comparable differences were subsequently observed with pure carcinogens, such as benzpyrene and methylcholanthrene (see Hartwell, 1951), and when so potent a carcinogen as 9,10-dimethyl-1,2-benzanthracene was employed, even the guinea-pig's skin proved responsive (Berenblum, 1949).

The commonly accepted pattern of response to *skin carcinogenesis* (in decreasing order) is mouse, rabbit, rat, fowl, guinea-pig. This is not the case, however, with all carcinogens. For instance, while tar is highly carcinogenic for skin both in the mouse and rabbit, benzpyrene (one of its constituents) is potent for the mouse but weak for the rabbit (Oberling and Guerin, 1947) while certain other tar fractions are very potent for the rabbit but not for the mouse (Berenblum and Schoental, 1947b).

When tested for *sarcoma production by subcutaneous injection*, the order for species response is very different from the above. The rat, so refractory

to skin carcinogenesis, develops subcutaneous sarcomas even more readily than the mouse (see reviews: Cook and Kennaway, 1938, 1940), and the fowl (Burrows, 1933) and other birds (Duran-Reynals *et al.*, 1945) are also highly responsive. Even the guinea-pig, the most refractory animal for skin carcinogenesis, readily develops sarcomas provided the dose injected is large enough (Berenblum, 1949; Russell and Ortega, 1952); on the other hand, the rabbit, one of the most susceptible to skin carcinogenesis, fails altogether to respond to subcutaneous injections of a carcinogen (Berenblum, 1949).

Variability in species response to *remotely acting carcinogenesis* is also quite pronounced. Estrone increases the mammary tumor incidence in the rat (McEuen, 1939) as well as in the mouse (Lacassagne, 1932), but has no such action in the rabbit, guinea-pig, dog, or monkey (see review: Gardner, 1947); in the guinea-pig, sub-serosal, fibroid-like growths develop in the uterus and abdominal viscera (Lipschutz and Iglesias, 1938; Lipschutz, 1950), and in hamsters, liver and renal tumors are produced (Kirkman, 1952). While *o*-aminoazotoluene is readily carcinogenic for the liver of the mouse and rat (see Shear, 1937), *p*-dimethylaminoazobenzene is very potent for the rat (Kinosita, 1937; Rusch *et al.*, 1945a), but only weakly carcinogenic for the mouse (Andervont *et al.*, 1944). Bladder tumor production with β-naphthylamine is easily achieved in the dog (Hueper *et al.*, 1938), but only after a long latent period in the rat (100 weeks) and rabbit ($5\frac{1}{2}$ years) (Bonser *et al.*, 1951), and not at all in the ferret, hamster, and guinea-pig (Bonser and Jull, 1952). With acetylaminofluorene, species influences manifest themselves as differences in the organs affected (Wilson *et al.*, 1941; Armstrong and Bonser, 1947; Foulds, 1947; Bonser and Green, 1950).

Differences in response also occur among the various inbred strains of the same species (mouse). Thus, the average latent period for skin tumor induction by tar painting ranged from 13.5 weeks in IF mice (a strain specially bred for rapid response to skin carcinogenesis) to 22 weeks in "White Label" mice (Bonser, 1938). (Early wart formation in IF mice was not, however, associated with a comparably rapid development of malignancy.) Similarly, with benzpyrene painting, the latent period ranged from 17.7 weeks in IF mice to 34 weeks in CBA mice. (In unpublished experiments by the author, the average latent period, with benzpyrene painting, was 30 weeks in C mice, 32 weeks in dba mice, 34 weeks in A mice, 42 weeks in C3H mice, and no neoplastic response after 38 weeks in C57 black mice.) With methylcholanthrene, the latent period was 10.5 weeks in IF mice and 20 weeks in CBA mice (Bonser, 1938); and similar differences were obtained when comparing New Buffalo and CBA mice (Cowdry and Suntzeff, 1944). In contrast to the unresponsiveness of

C57 black mice to benzpyrene painting (see above), these were found to be more responsive than C3H mice to dibenzanthracene painting (Lauridsen and Eggers, 1943).

Strain differences also occur with sarcoma production by subcutaneous injection (see review: Fieser, 1938; also Andervont, 1938; Bonser, 1940; Burdette and Strong, 1943), but responsiveness to skin carcinogenesis and to subcutaneous carcinogenesis do not run parallel. Thus, the IF strain, which is most responsive to skin painting, is relatively insensitive to subcutaneous injection, while C3H mice, which are relatively insensitive to skin painting, are among the most susceptible to subcutaneous carcinogenesis (see Table I).

TABLE I

Differences in Susceptibility to Representative Examples of Spontaneous and Induced Tumor Development in Inbred Strains of Mice

	Spontaneous Tumors		Induced Tumors	
STRAIN	Mammary	Lung	Skin	Subcutaneous
C_3H	Very high	Low	Fairly low	High
A	High	Very high	Medium	Fairly high
White Label	Medium	Low	Fairly low	Medium
C	Low	Medium	Fairly high	Medium
IF	Very low	Low	High	Medium
C57 black	Very low	Very low	Low	Medium

Differences in lung tumor incidence, among the various strains, in response to subcutaneous injection of dibenzanthracene, are even more striking, strain A mice being exceptionally susceptible, and C57 black mice, particularly resistant (Andervont, 1937a). These differences run parallel to those affecting the spontaneous incidence of lung tumors in these strains, suggesting that the effect of the carcinogen constitutes merely an accentuation of a spontaneous tendency. (The view that all forms of induced carcinogenesis are mere manifestations of an acceleration of a spontaneous tendency (Lefèvre, 1945) is not generally accepted, however.) Cross-breeding experiments suggest (Heston, 1940) that multiple genetic factors influence lung tumor carcinogenesis. That the genetic susceptibility resides in the lung tissue itself, has been elegantly demonstrated by transplanting lung tissue from susceptible and nonsusceptible strains, respectively, into hybrids of the two, and observing a higher tumor incidence in the former than in the latter grafts after the hosts received intravenous injections of dibenzanthracene (Heston and Dunn, 1951), or of urethane (Shapiro and Kirschbaum, 1951).

Strain differences in response to remotely acting carcinogenesis have been observed with azo compounds (Andervont *et al.*, 1942; Law, 1941a),

with acetylaminofluorene (Armstrong and Bonser, 1947; Dunning *et al.*, 1947), and particularly with estrone (Lacassagne, 1938a; see also Gardner, 1947), the latter being complicated by the additional participation of the "milk agent" (Bittner, 1942).

The special breeding of strains for high and low sensitivity to carcinogenic response has been approached from at least three different angles: (1) by straightforward selection for high and low response to skin carcinogenesis (Bonser, 1938, 1940); (2) by selection for resistance to local tumor induction (by methylcholanthrene) so that remote tumors, e.g., hepatomas, might have a better chance of developing (Strong, 1944); and (3) by the continued breeding of mice *kept under the influence of methylcholanthrene treatment*, with the object of inducing germinal mutations, involving an enhanced susceptibility to spontaneous and induced tumor development of unusual types (e.g., gastric carcinoma in NHO mice) (Strong, 1949a). (Schabad (1929) previously observed that the tendency toward an increased lung tumor incidence in tarred mice was transmitted to untarred offspring.)

From the results, summarized above, of locally acting and remotely acting carcinogenesis in different species and strains of animals (see also Table I), the following conclusions may be reached.

1. Genetic factors influence the response to extrinsic carcinogenic action, the genetic differences being, on the whole, more pronounced with respect to remote than to local carcinogenesis.

2. As is the case with spontaneous tumor development, the genetic influences on induced carcinogenesis operate independently on the different tissues of the body.

3. In view of such genetic differences in response, and because of the absence of any correlation in respect of the different tissues toward one and the same carcinogen, the term "carcinogenic potency" can have no absolute value, and cannot, therefore, be correlated quantitatively with other (physical or chemical) properties which the carcinogens may possess (see Daudel and Daudel, 1950; Berenblum, 1951b; Coulson, 1953).

IV. Influence of Age, Sex, and Hormonal Factors

Age, sex, pregnancy, castration, and other forms of hormonal influence, play an insignificant role in determining the response to carcinogenic action. There are, however, special cases where the hormonal influence is important, and under some conditions, even decisive.

For sarcoma induction by subcutaneous injection of carcinogenic hydrocarbons, age has practically no influence (Dunning *et al.*, 1936), though Shimkin (1939) found young animals somewhat more responsive, while Brunschwig and Tschetter (1937) found, if anything, an opposite tend-

ency. For skin carcinogenesis, the influence of age is slight (Cowdry and Suntzeff, 1944), except for the first few hours after birth, when the skin seems unresponsive to methylcholanthrene carcinogenesis (Suntzeff *et al.*, 1947), a result attributed to the absence of sebaceous glands and hair follicles at that stage of development. Against this explanation, the following may be cited: (*a*) carcinogenic hydrocarbons can penetrate newborn skin (Setälä and Ekwall, 1950), (*b*) the carcinogens persist on the skin far beyond the period (24 hours) during which skin appendages remain undeveloped (Hieger, 1936; Heidelberger and Weiss, 1951), and (*c*) even embryonic skin responds to methylcholanthrene carcinogenesis, as shown by implantation of embryonic skin plus carcinogen into adult animals (Greene, 1945; Rous and Smith, 1945). In the case of remote carcinogenesis in the lung by urethane, very young mice are actually *more* responsive (Rogers, 1951).

Sex plays no significant role in skin carcinogenesis in most strains of mice (Bonser, 1940), though in some strains, males are somewhat more responsive than females (Kreyberg, 1935). Boyland and Warren (1937) found no influence of sex on subcutaneous carcinogenesis with methylcholanthrene, though a significantly higher incidence of tumors was observed in males by Leiter and Shear (1943) when marginal doses were used. With remote carcinogenesis, males are more responsive to acetylaminofluorene with respect to tumor production in the liver (Bielschowsky, 1944; Leathem, 1951), and bladder (Armstrong and Bonser, 1947; Foulds, 1947), while mammary gland carcinogenesis by acetylaminofluorene is more effective in females (Bielschowsky, 1944; Foulds, 1947). With azo compounds, the results differ according to the compound used, *o*-aminoazotoluene being more effective in females (Andervont *et al.*, 1942) and 3′-methyl-4-dimethylaminoazobenzene in males (Rumsfeld *et al.*, 1951). In short, no consistent influence of sex on carcinogenesis is discernible.

Similarly, pregnancy, castration, and splenectomy have no influence on tar carcinogenesis (see review: Woglom, 1926), and sarcoma production in mice by methylcholanthrene is also unaffected by castration (Boyland and Warren, 1937); but with acetylaminofluorene, carcinogenesis of mammary tissue in female rats is inhibited by castration (Bielschowsky, 1944).

In a study of hyperactivity (by injection of hormones) and hypoactivity (by removal of endocrine glands), in relation to methylcholanthrene acting subcutaneously in rats (Smith *et al.*, 1942), no influence was observed from excess or deficiency of estrone, progesterone, testosterone, deoxycorticosterone, adrenalin, thyroid, insulin, pituitary, or prolactin. A slight increase in tumor rate resulted from injection of gonadotropins, and a slight decrease, from injection of the cortin group of compounds. (Had marginal, instead of optimal, doses of carcinogen been used, the results

might have been more convincing.) Skin carcinogenesis is augmented by concurrent application of estrone, according to Gilmour (1937), and somewhat delayed, according to Paletta and Max (1942); remote carcinogenesis with acetylaminofluorene seems to be augmented both by estrogens and androgens (Cantarow *et al.*, 1946).

It is perhaps not surprising that the influence of hormonal imbalance on carcinogenesis should be most striking when the tumor-inducing process affects the endocrine glands themselves (Gardner, 1947; 1948). The best known example of this is, of course, the decisive role that estrone plays in the development of mammary tumors in mice, in conjunction with a favorable genetic susceptibility and the presence of the milk agent (Bittner, 1942). Tumor development in the lungs (lymphosarcoma), adrenal medulla, and ovary, after injection of pituitary growth hormone (Moon *et al.*, 1950a,b,c), does not occur in hypophysectomized animals (Moon *et al.*, 1951). Hypophysectomy also inhibits subcutaneous carcinogenesis with methylcholanthrene (Moon *et al.*, 1952) and liver carcinogenesis with azo dyes (Griffin *et al.*, 1953).

An example of hormonal imbalance being itself responsible for tumor development is also afforded by the transplantation of the ovaries into the spleen. The physiological consequence of such transplantation is that the ovarian hormones are carried from the new location through the portal circulation to the liver, where they are destroyed (Zondek, 1941), thus never reaching the pituitary gland; this leads to oversecretion of gonadotropic hormones, which continue to stimulate the grafted ovarian tissue. The pathological consequence of this is the ultimate development of neoplasia of the transplant (Biskind and Biskind, 1944; Li and Gardner, 1947). Similarly, testicular grafts into the spleen of castrated rats may become neoplastic (Biskind and Biskind, 1945). Another example of hormonal imbalance acting as a carcinogenic stimulus has been demonstrated in *ce* mice, in which gonadectomy immediately after birth led to the development of adrenal carcinoma in later life (Woolley and Little, 1945a,b).

It is tempting to imagine (Gardner, 1947; Hertz, 1951) that hormonal imbalance may also play a part, indirectly, in chemical carcinogenesis of endocrine glands, e.g., that carcinogenesis of the thyroid gland by the action of thiourea may be due to interference with the thyrotropic mechanism of the pituitary, rather than to a direct action on the thyroid gland (see also: Bielschowsky and Griesbach, 1950).

V. Dietary Factors Influencing Carcinogenesis

A clear distinction must be made between those dietary factors that influence only certain types of tumor induction and those that influence carcinogenesis in general.

Of the former, limited type of influence, the most interesting is that associated with hepatoma production by azo compounds. The ease with which they were first produced in rats kept on a relatively poor diet of unpolished rice (Sasaki and Yoshida, 1935), and the difficulties experienced with animals on more balanced diets (see Burk and Winzler, 1944; Orr, 1947), soon led to the discovery of "protective factors" such as yeast and liver. From further studies in which known components were added to purified diets (Kensler *et al.*, 1941; Miner *et al.*, 1943), both protective (anticarcinogenic) and augmenting (procarcinogenic) influences could be ascribed to individual components. While pyridoxine increased liver tumor induction by azo compounds (Miner *et al.*, 1943), riboflavin prevented their appearance (Kensler *et al.*, 1941), especially in the presence of casein, an effect antagonized by biotin (du Vigneaud *et al.*, 1942) and egg-white (the latter, by virtue of its avidin content). It is interesting to note, however, that hepatoma production by acetylaminofluorene is not inhibited by riboflavin (Engel and Copeland, 1952). The protective action of riboflavin, in the case of azo dyes, is probably associated with the participation of flavin-adenine-dinucleotide in the cleavage of the azo linkage (Kensler, 1949; Mueller and Miller, 1950).

Other components of the vitamin B complex (inositol, niacin, nicotinamide, choline, *p*-aminobenzoic acid, folic acid, etc.) on azo dye carcinogenesis are less effective in influencing liver carcinogenesis (Various Authors, 1947b; Kensler, 1952; Harris and Clowes, 1952). Rather uniquely, liver tumors can be induced by choline deficiency per se (Copeland and Salmon, 1946), an effect also antagonized by riboflavin (Schaefer *et al.*, 1950). (For the role of azo compounds and choline deficiency on enzymic activity of the liver, see review: Kensler, 1952.) In guinea-pigs, induced scurvy shortens the induction time of subcutaneous carcinogenesis with methylcholanthrene (Russell *et al.*, 1952).

As riboflavin, and the other vitamin B factors mentioned above, only influence liver carcinogenesis, without any apparent effect on carcinogenesis of the skin (Gordonoff and Ludwig, 1938; Tannenbaum and Silverstone, 1952) or subcutaneous tissues (Strong and Figge, 1946), they cannot be intimately connected with the general problem of tumor pathogenesis. [For reviews on the influence of diet on stomach carcinogenesis, see Klein and Palmer (1940), Sugiura (1942), Burk and Winzler (1944), and Barrett (1946).]

A high fat content of the diet augments skin carcinogenesis by tar (Watson and Mellanby, 1930), polycyclic hydrocarbons (Jacobi and Baumann, 1940; Tannenbaum, 1942b), and ultraviolet rays (Baumann and Rusch, 1939), as well as liver carcinogenesis by azo dyes (Opie, 1944). Its influence on skin carcinogenesis is attributed to the fatty acid components

(Lavik and Baumann, 1941), though with liver carcinogenesis, the situation appears to be more complex, since the effect varies with the type of fat used (Miller, 1947). In contrast to these results, sarcoma production by subcutaneous injection of carcinogens remains unaffected, or is even inhibited, by a high fat content of the diet (Tannenbaum, 1942b).

The influence of proteins (Tannenbaum and Silverstone, 1949; 1953), or more specifically of their various amino acid constituents, on carcinogenesis, has been less extensively studied. A low cystine diet completely inhibits the development of spontaneous mammary cancer in susceptible strains of mice (White and Andervont, 1943), an effect partly reversed by implantation of stilbestrol pellets (White and White, 1944). In a strain in which leukemia develops in about 90% after skin painting with methylcholanthrene, a low cystine diet reduced the incidence to about 10% (White *et al.*, 1944b), whereas a lysine-restricted (White *et al.*, 1944b) or tryptophan-restricted diet (White *et al.*, 1947) did not have any such inhibitory effect. For bladder tumor production in rats with acetylaminofluorene, a diet low in proteins and vitamins is, on the contrary, a necessary condition (Strombeck and Ekman, 1949).

The failure to discover any dietary component which could influence all forms of carcinogenesis, is all the more surprising in the light of the fact that caloric restriction per se is profoundly inhibitory for a wide range of tumor (Tannenbaum, 1940; 1942; 1947; White *et al.*, 1944a; Tannenbaum and Silverstone, 1953).

Such inhibition by caloric restriction operates for both spontaneous and induced tumor genesis. The spontaneous group includes tumors of the breast (Tannenbaum, 1940, 1942a; White *et al.*, 1944a), lung (Larsen and Heston, 1945), liver (Tannenbaum, 1945) and leukemia (Saxton *et al.*, 1944). Experimentally induced tumors include those of the skin, painted with carcinogenic hydrocarbons (Tannenbaum, 1940, 1942a; White *et al.*, 1944a) or irradiated with ultraviolet rays (Rusch *et al.*, 1945c), of subcutaneous tissues (Tannenbaum, 1940, 1942a; Rusch *et al.*, 1945b), as well as leukemia induced by carcinogenic skin painting (White *et al.*, 1944a).

Such underfed mice, though remaining smaller than the controls, appear normal and healthy, and actually live longer. The tumor inhibition occurs not only when all the components of a balanced diet are equally reduced (in which case, some specific components might, in fact, fall below a critical level), but also when the proteins, fats, and vitamins are maintained at the same level as in the controls, with the caloric restriction achieved by reduction of carbohydrates only (Tannenbaum, 1942a). The inhibition must, therefore, be attributed to caloric restriction per se.

The suggestion (White *et al.*, 1944a) that the resulting anestrus is re-

sponsible for the reduced mammary tumor development following caloric restriction has been questioned (Tannenbaum, 1942a) on the grounds that undisputed hormonal inhibition, by ovarietomy, is only effective when the operation is performed in infancy (Lathrop and Loeb, 1916), while caloric restriction is effective even when begun late in life. (For a detailed discussion of the mechanism of inhibition of carcinogenesis by dietary restriction, see Tannenbaum and Silverstone, 1953.)

The depression of epidermal mitotic activity in animals maintained on a restricted diet (Bullough, 1949b) provides an alternative approach to the study of the mechanism of inhibition (see below). The possibility of the diet influencing carcinogenesis through a hormonal disturbance, is discussed by Morris (1952).

VI. Effect of Solvents on Carcinogenesis

In the early studies on skin carcinogenesis with tar, the role of solvent was considered (*a*) as a simple diluent, to render the viscous tar more manageable for skin painting (and, sometimes, to reduce its irritative qualities); (*b*) as a differential solvent, to extract the active components, either for providing a more potent preparation than the crude material or as a first stage in fractionation; and (*c*) as an adjuvent, with the intention of increasing the irritative quality of the tar, in the mistaken belief that skin carcinogenesis depended on nonspecific irritation. On the whole, the choice of solvent used did not materially affect the neoplastic response. Even as diluent, the effect was not pronounced till the concentration of the tar was reduced to below 10%, when the carcinogenic activity began to fall off rapidly (Hieger, 1936).

With the use of pure compounds (dibenzanthracene, benzpyrene, methylcholanthrene, etc.) for skin carcinogenesis, the choice of solvent became narrowed down to one that would dissolve these relatively insoluble compounds in as high a concentration as possible (e.g., up to 0.5 or 1%), without the solvent producing any effect of its own on the skin. Of the volatile solvents used, acetone comes nearest to these requirements (Orr, 1938), though benzene is still widely used, despite its toxicity and slightly irritative action on the skin.

The commonly used solvents for skin painting experiments (acetone, benzene, ethanol, ether, chloroform, etc.) have little specific influence on the carcinogenic potency of carcinogenic hydrocarbons, though minor variations have been reported. A pronounced inhibition of skin carcinogenesis was found when lanolin served as solvent for methylcholanthrene, at a concentration of carcinogen (0.3%) which was adequate with benzene as solvent (Simpson and Cramer, 1943, 1945; Simpson *et al.*, 1945). This was, at the time, attributed to a specific anticarcinogenic property of

the lanolin (Simpson and Cramer, 1945), but has since been shown to be due to a simple solubility effect (Berenblum and Schoental, 1947a), arising from the fact that lanolin is nonvolatile, so that the carcinogen remains on the skin *in its initial, low concentration*, whereas benzene, or any other volatile solvent, rapidly evaporates from the skin surface, leaving the carcinogen dissolved, *in a more concentrated form*, in the natural fats on the skin surface. A significant corollary of this is that for quantitative studies in skin carcinogenesis (for which a constant *effective* concentration of carcinogen is obviously desirable), a nonvolatile solvent is preferable to a volatile one (making allowance for the need of a higher concentration of carcinogen, when the nonvolatile solvent is used, to elicit a comparable response). Medicinal liquid paraffin (refined mineral oil, or petrolatum) has proved ideal for such quantitative studies (Berenblum and Shubik, 1947a,b).

Of special interest are the recent trials with polyethylene oxide (Stamer, 1945), polyethylene glycols, Carbowax, and other "associated colloids" (Setälä, 1949a,b) as solvents for carcinogenic hydrocarbons. These possess both lipoid-soluble and water-soluble properties, thereby, supposedly, facilitating the transfer of the water-insoluble hydrocarbons to the aqueous phase within the living cell (Setälä, 1949b; Ekwall and Setälä, 1948, 1950). Their use also has special significance in carcinogenic studies on the stomach, where the difficulty of penetrating the mucin barrier, at the surface of the glandular portion of the stomach, may account for the failure, in the past, of inducing adenocarcinomas of the stomach by feeding carcinogens (Klein and Palmer, 1940; Lorenz and Stewart, 1940). That carcinogens dissolved in such associated colloids do penetrate the mucosa of the glandular portion of the stomach, has been demonstrated by fluorescence microscopy (Ekwall *et al.*, 1951). However, adenocarcinoma of the stomach did not develop from prolonged administration of a carcinogen dissolved in such media (Saxen and Ekwall, 1950), nor with the addition of eugenol, which is a more drastic means of breaking down the mucin barrier (Hitchcock, 1952).

For sarcoma production by subcutaneous injections, the influence of solvent is exceptionally complicated, involving, in theory, the following possible mechanisms: (1) the solvent serving merely as diluent, determining the intensity of action of the carcinogen; (2) the solvent influencing the rate of absorption of the carcinogen into the neighboring cells; (3) the solvent influencing the rate of diffusion of the carcinogen away from the site of action; (4) changes in the effective concentration of the carcinogen, through a more rapid absorption of the solvent than of the carcinogen; (5) the solvent acting on the carcinogen itself, destroying it, or, by virtue of anti-oxidant properties, preserving it from rapid destruction *in situ;*

or alternatively enhancing carcinogenesis by facilitating a (supposedly) necessary metabolic conversion of the carcinogen; and (6) the solvent being itself carcinogenic, or influencing the responding tissues through cocarcinogenic or anticarcinogenic action. With so many possible variables, it is hardly surprising that the present state of knowledge about the influence of solvent on subcutaneous carcinogenesis should still be confusing (Rusch, 1944; Dickens, 1947; Peacock *et al.*, 1949).

In the past, the most commonly used media for subcutaneous injection of carcinogenic hydrocarbons were vegetable oils (Burrows, 1932; Schabad, 1935; Oberling *et al.*, 1936), paraffin (Haagensen and Krehbiel, 1936; Dunning *et al.*, 1936), lard (Burrows *et al.*, 1932; Andervont, 1934: Shear, 1936) and other animal fats (Peacock, 1933, 1935); also coarse suspensions (i.e., crystals of the carcinogen moistened with glycerol) (Shear, 1938) or incorporation of the carcinogen in cholesterol pellets (Shear, 1936; Ilfeld, 1936; Shimkin and Wyman, 1947); and colloidal suspensions in water (Berenblum and Kendal, 1934; Boyland and Burrows, 1935), serum (Andervont and Lorenz, 1937), bile salts (Shear, 1936), etc. Because of the variability in composition of natural fats and the consequent differences in neoplastic response (Shimkin and Andervont, 1940; Leiter and Shear, 1943), these have been largely superseded by the use of synthetic tricaprylin as solvent (Shimkin and Andervont, 1940; Bryan and Shimkin, 1943), or by other inert solvents of constant composition. The early studies served the useful purpose, nevertheless, of drawing attention to physical, chemical, and biological factors that modified neoplastic response.

Apart from minor variations in response with vegetable oils, lard, paraffin, or synthetic glycerides as solvents (Shimkin and Andervont, 1940; Leiter and Shear, 1943), the first example of a strong influence of solvent on subcutaneous carcinogenesis was the dramatic inhibition by homologous fats, observed in rats, with rat fat as solvent (Watson, 1935), in chickens, with egg yolk or chicken fat as solvent (Peacock, 1935), and in mice, with mouse fat as solvent (Peacock and Beck, 1938; Morton and Mider, 1939), or when the carcinogen was injected as a powder or dissolved in ether, and, therefore, ultimately became dissolved in the animal's own fat (Peacock and Beck, 1938). (Failure to observe such inhibition with homologous fats (Shimkin and Andervont, 1940; Oberling *et al.*, 1939b) has been attributed (Dickens, 1947) to the use of excessive doses of carcinogen.)

Since the carcinogen (benzpyrene) rapidly disappeared from the site of injection when homologous fat was used, and very much more slowly when olive oil served as solvent (evidence of disappearance being judged visually by local absence of fluorescence at autopsy), the inhibition was

attributed to inadequate duration of action (Peacock and Beck, 1938). Though subsequent experiments, based on quantitative estimations of carcinogen remaining *in situ* after various intervals, indicating an opposite trend, namely, that a slower rate of elimination was associated with a lower carcinogenic activity (Weil-Malherbe and Dickens, 1946), this does not necessarily invalidate the original conclusion, since it is not the rate of elimination *in the presence of residual carcinogen*, but the time taken for *complete* elimination, that determines the duration of action, and presumably, therefore, the tumor inducing efficiency (Peacock *et al.*, 1949). Probably both opposite trends operate, the resulting effect depending on (a) how much carcinogen was present at the start, and (b) how rapidly it subsequently disappears.

From a comparative study of the solvent-serum distribution coefficient for different solvents used for carcinogenic studies, in relation to neoplastic response, Strait *et al.* (1948) concluded that a persistent, slow, liberation of carcinogen was most effective for carcinogenic action.

Based on the idea that the inhibitory effect of homologous (mouse) fat might be due to a specific anticarcinogenic constituent in it, comparative tests were carried out with various fractions (Dickens and Weil-Malherbe, 1942, 1946; Weil-Malherbe and Dickens, 1946). While a neutral fraction, partially purified from phospholipids, was still inhibitory, further purification yielded a fraction which was no longer so, thus suggesting that the phospholipids were responsible. However, though purified phospholipids (tested in various concentrations in tricaprylin, as solvent for the carcinogen) did prove inhibitory *in high doses* (Dickens and Weil-Malherbe, 1946), no such effect was observed with doses more comparable to those normally present in mouse fat (Weil-Malherbe, 1946); while cholesterol, if anything, actually augmented carcinogenic action. In any case, this could not explain why a similar inhibition does not occur heterologously.

Since phospholipids are anti-oxidants and since cod liver oil, which is particularly rich in unsaturated fats, produced no inhibitory effect when acting as solvent of carcinogens (Leiter and Shear, 1943; Dickens and Weil-Malherbe, 1946), whereas hydrogenated cod liver oil was inhibitory, it has been suggested (Weil-Malherbe and Dickens, 1946; Dickens, 1947) that anti-oxidants interfere with carcinogenesis by preventing the metabolic oxidation of the carcinogen, supposedly necessary for the carcinogenic action. It is hard to understand, however, how this could explain the difference in action between homologous and heterologous fats.

It may be that emphasis on the *homologous* nature of the inhibitory fats has been given exaggerated importance, seeing that *heterologous* fats,

except for lard, have not been sufficiently investigated as necessary controls. The problem might, indeed, turn out to be one of animal versus vegetable fats, with lard as an anomalous exception. In this connection, it may be noted that ox brain lipids are inhibitory for mice (Dickens and Weil-Malherbe, 1942), while lard itself contains both inhibitory and augmenting components, when tested in mice (Shear, 1936; Leiter and Shear, 1943; Shimkin and Andervont, 1940).

The balance of evidence points to the conclusion that a solvent that encourages too rapid a rate of elimination of a carcinogen, especially when the initial dose of carcinogen is small, functions as an inhibitory agent. This does not preclude the possibility of anti-oxidants stabilizing a relatively labile carcinogen (such as 9,10-dimethyl-1,2-benzanthracene) *in situ* (Chevallier *et al.*, 1946); nor does it preclude the possibility of anti- or cocarcinogenic effects in certain situations. That the latter may operate subcutaneously is demonstrated by the cocarcinogenic effect of kieselguhr (Lacassagne, 1933), kaolin, or silica (Burrows *et al.*, 1937) on x-ray carcinogenesis, where the question of rate of elimination of carcinogen obviously does not come into play.

VII. Irritation and Carcinogenesis

Scarification of the mouse's skin before each application of tar was first performed with the dubious objective of facilitating better penetration of the carcinogen. The claim that carcinogenesis was thereby speeded up (Deelman, 1923; Teutschlaender, 1923) was not generally confirmed, however (see Roussy *et al.*, 1924; Ludford, 1929). As distinct from acceleration of carcinogenesis, the observed tendency for tar tumors in mice to become preferentially located at the edges of deep incisions (Deelman and van Erp, 1926) was subsequently confirmed (Pullinger, 1943; Kline and Rusch, 1944), and found to operate far more effectively in rabbits (MacKenzie and Rous, 1941; see below). Many other forms of irritation have since been tested in conjunction with tar or carcinogenic hydrocarbons, with varied results (see review, Berenblum, 1944). These were very complicated and discordant when the irritants were made to act *concurrently* with the carcinogen, leading, in some cases, to augmentation (cocarcinogenic action), in others, to inhibition (anticarcinogenic action), and in the majority of cases, to no influence on carcinogenesis one way or the other.

Cocarcinogenic action on *skin* has been observed with ultraviolet radiation (Findlay, 1928; Dormanns, 1934; but cf. negative results by Kohn-Speyer, 1929; Taussig *et al.*, 1938), heat (Derom, 1924; Raposo, 1928; Lauridsen and Eggers, 1943; but see negative results by Choldin, 1930; Brunschwig *et al.*, 1937; des Ligneris, 1940), estrone (Gilmour, 1937), a basic, noncarcinogenic, fraction of tar (Sall *et al.*, 1940), croton

oil (Berenblum, 1942a,b), *o*-aminoazotoluene, and chlorophyll (Rosicki and Hatschek, 1943). Cocarcinogenic effects have also been obtained with low doses of carcinogen *subcutaneously injected* concurrently with the basic fraction of tar (Sall *et al.*, 1940) and with croton oil (Klein, 1951).

Anticarcinogenic action on skin was observed with sulfur mustard and some of its analogues, cantharidin (Berenblum, 1929, 1935), *p*-thiocresol (Reimann and Hall, 1936) phenolic fractions of tar (Shear, 1938; Cabot *et al.*, 1940), monochloracetone and related compounds (Crabtree, 1940a), heptaldehyde (Carruthers, 1940), strong sunlight (Doniach and Mottram, 1940), organic acid chlorides (Crabtree, 1941b), vitamin A (Rosicki and Hatschek, 1943), BAL:[2,3-dimercaptopropanol (Crabtree, 1948)], podophyllotoxin (Berenblum, 1951a), Indoleacetic acid (Berenblum and Haran, unpublished results), and various noncarcinogenic or weakly carcinogenic hydrocarbons (Lacassagne *et al.*, 1945; Riegel *et al.*, 1951).

In view of these divergent results, with different irritants, irritation *per se* cannot be a decisive factor for cocarcinogenic or anticarcinogenic action.

With carcinogens and "irritants" acting concurrently *from a distance*, the results are even more complicated. The effects of acetylaminofluorene and azo dyes on liver carcinogenesis are additive and even synergistic (MacDonald *et al.*, 1952); yet combination of thiouracil with acetylaminofluorene (Paschkis *et al.*, 1948) or with azo dyes (Harris and Clowes, 1952), nitrogen mustard with azo dyes (Griffin *et al.*, 1951), methylcholanthrene with azo dyes (Richardson and Cunningham, 1951), or alloxan and azo dyes treatment (Salzberg and Griffin, 1952) inhibits liver carcinogenesis; while addition of urethane, itself mildly carcinogenic for the liver, to azo dye treatment has no effect either way (Jaffé, 1947b). Addition of methylcholanthrene (Jaffé, 1947a) or acetylaminofluorene (Jaffé, 1947b) to urethane treatment is also without effect on lung tumor incidence; while mammary tumor induction with estrone is augmented by simultaneous administration of methylcholanthrene (Dmochowsky and Orr, 1949), but not with simultaneous administration of acetylaminofluorene (Cantarow *et al.*, 1948). Croton oil fails to augment skin carcinogenesis when applied together with *p*-dimethylaminoazobenzene (a remotely acting carcinogen which is at the same time locally carcinogenic for the skin) (Kirby, 1948a), but has a slightly augmenting effect with acetylaminofluorene (Kirby, 1948b). (For synergism of leukemia-inducing agents, and factors favoring and inhibiting leukemogenesis, see review, Kirschbaum, 1951.) It is clear that the available data are inadequate to provide even a tentative hypothesis to explain the varied effects of concurrently administered remotely acting carcinogens. (But see Miller *et al.*, 1952, on a possible relation between the inhibitory action of carcinogenic hydro-

carbons on azo dye carcinogenesis and the capacity of the liver to metabolize the azo compounds.)

Reverting to locally acting, *anticarcinogenic* influences, that of sulfur mustard has been shown to be due to a local action on the skin, and not to a chemical interaction with the carcinogen, nor to any systemic influence (Berenblum, 1929, 1931). This probably applies generally to anticarcinogenic agents acting on the skin concurrently with a carcinogen. An attempt to correlate anticarcinogenic action with inhibition of glycolysis, failed when extended to related compounds of sulfur mustard (Berenblum *et al.*, 1936). The idea was later revived by Crabtree (1940b) in connection with monochloracetone and related compounds, but since these compounds were only moderately anticarcinogenic, and in higher concentrations even displayed cocarcinogenic properties (Crabtree, 1941a), the alleged association seems questionable. An extension of the hypothesis, implicating an inhibition of the S-metabolism in the cell as responsible for anticarcinogenic action (Crabtree, 1948), while consistent with the observation that BAL is anticarcinogenic (see above), and indirectly supported by studies *in vivo* (White and White, 1939) and *in vitro* (Calcutt, 1949) on carcinogenic hydrocarbons as —SH inhibitors, is too speculative to be accepted without further corroboration.

The inhibitory effect of a noncarcinogenic or weakly carcinogenic hydrocarbon, applied concurrently with a potent one, has been attributed to a competitive affinity for the same receptor within the cell (Lacassagne *et al.*, 1945), a hypothesis improbable a priori, since tar, which is rich in such "competitive" noncarcinogenic hydrocarbons, is nevertheless potently carcinogenic. The results of Lacassagne *et al.* (1945), based on small numbers of animals, were only partly confirmed when repeated on a more adequate scale and extended to other hydrocarbons (Riegel *et al.*, 1951; Hill *et al.*, 1951). Thus, while the anticarcinogenic action of 1,2,5,6-dibenzfluorene was confirmed, no such action was observed with chrysene, nor did naphthalene, fluorene, or 1,2,7,8-dibenzfluorene prove to be anticarcinogenic, while anthracene exhibited, if anything, cocarcinogenic activity. When two potent carcinogens were applied together to the skin, the latent period was somewhat longer than with the more potent of the two acting alone (Hill *et al.*, 1952); yet when two carcinogens were injected together subcutaneously (Steiner and Falk, 1951), the effect was more often additive (i.e., cocarcinogenic) than inhibitory (or anticarcinogenic). It would seem, therefore, from the evidence so far available, that the anticarcinogenic activity of 1,2,5,6-dibenzfluorene, etc., involves a more specific mechanism than that postulated by Lacassagne *et al.* (1945). But the nature of the mechanism, whether for these hydrocarbons, or for anticarcinogens in general, is still obscure.

The mechanism of *cocarcinogenic* action lent itself more readily to analysis, by segregating the available data from the literature according to whether the irritant acted concurrently with the carcinogen (see above), or was administered beforehand, or was begun after cessation of the carcinogenic treatment (Berenblum, 1944). Before passing on to the two latter categories, attention must be drawn to the situation in which the two agents, acting during separate periods, are both carcinogenic.

That the carcinogenic process can be begun by one carcinogenic hydrocarbon and completed by another, was demonstrated by Hieger (1936) and later confirmed on a more quantitative basis (Rusch *et al.*, 1942; Lavik *et al.*, 1942), thus indicating that the different hydrocarbons probably have an identical mechanism of action (but see below, Stages of Carcinogenesis). In striking contrast to this, no such additive effects were obtained when ultraviolet irradiation was followed by painting with a chemical carcinogen, or vice versa (Rusch *et al.*, 1942).

When a carcinogen and a noncarcinogenic agent are applied to the skin during separate periods, the results are not only different from those operating when the two act concurrently (see above), but vary according to whether the irritant is applied before the commencement of the carcinogenic treatment or after its cessation (see review, Berenblum, 1944). With *pretreatment* of the irritant, carcinogenesis remains virtually unaffected; with *posttreatment*, an enhancement of tumor induction occurs in many cases, e.g. when the irritation consists of skin incision (Deelman and van Erp, 1926; Pullinger, 1943; 1945; MacKenzie and Rous, 1941; Meyenburg and Fritzsche, 1943; Linell, 1947), chronic mechanical trauma by light brushing (Riley and Pettigrew, 1945), cauterization (Rondoni and Corbellini, 1938), freezing (Berenblum, 1930), gamma and beta rays (Mottram, 1937, 1938), or by painting the prepared skin with allyl*iso*thiocyanate (Sobolewa, 1936), oleic acid (Twort and Twort, 1939), turpentine (Rous and Kidd, 1941), chloroform (Friedewald and Rous, 1944a), naphthoquinone (Kline and Rusch, 1944), iodoacetic acid, chloracetophenone (Gwynn and Salaman, 1951), and, most effectively (in mouse skin), croton oil, or its active component, croton resin (Berenblum, 1941b; Mottram, 1944a; Kline and Rusch, 1944; Berenblum and Shubik, 1947a; Bielschowsky and Bullough, 1949; Klein, 1952).

This capacity of certain noncarcinogenic irritants to "precipitate" tumor development in a tissue previously "prepared" by a limited period of carcinogenic treatment, has been variously described as "epicarcinogenic action" (Berenblum, 1941b), "developing factor" (Mottram, 1944a), "stage of development or formation" (Tannenbaum, 1944), and "promoting factor" (Friedewald and Rous, 1944a,b); while the initial, preparative, action by the carcinogen has been designated by different

authors as "precarcenogenic action" (Berenblum, 1941b), "specific cellular reaction" (Mottram, 1944a,b), "stage of preparation or initiation" (Tannenbaum, 1944), and "initiating action" (Friedewald and Rous, 1944a,b). Rous's nomenclature of "initiating action" and "promoting action" for the two phases of carcinogenesis has now been generally accepted (Berenblum and Shubik, 1947b).

In spite of the wide range of promotors, described above, not all irritants are capable of producing such an effect; moreover, the action is often tissue and species specific. Thus, croton oil, the most potent promoting agent for the mouse's skin, is inactive for the rat, rabbit, or guinea-pig's skin (Shubik, 1950a), while wound healing, which is highly effective for the rabbit skin (MacKenzie and Rous, 1941) is only slightly effective for mouse skin (Pullinger, 1943). Among the irritants that fail to act as promoting agent when tested on mouse skin, are sulfur mustard (Berenblum, 1931), liquid paraffin, lanolin (Twort and Twort, 1939), ultraviolet irradiation (Rusch *et al.*, 1942), acridine, fluorene, phenanthrene, castor oil, ricinoleic acid, glyceryl monoricinoleate, oleic acid, silver nitrate (Shubik, 1950a), acetic acid, cantharidin, podophyllin resin, mustard oil, and iodoacetamide (Gwynn and Salaman, 1951), pyrene, atabrin (Bernelli-Zazzera, 1952), indolacetic acid, indolpropionic acid, indolbutyric acid, and methylindol acetate (Berenblum and Haran, unpublished results).

VIII. Initiating and Promoting Action as Independent Stages of Carcinogenesis

From the above, it is evident that croton oil is a powerful promoting agent for the mouse, giving rise to tumors when repeatedly applied to skin which had previously been treated with a carcinogen for 8 weeks (Berenblum, 1941b) or even once only (Mottram, 1944a; Berenblum and Shubik, 1947a,b). Yet, croton oil by itself is not carcinogenic (Berenblum, 1941a; Klein, 1952), and, when applied for 26 weeks *prior to the treatment with the carcinogen,* does not speed up tumor production (Berenblum, 1941b). The fact that croton oil can complete the carcinogenic process but cannot initiate it indicates that these two phases of carcinogenesis have independent mechanisms (Berenblum, 1941b).

A similar conclusion was reached from a study of the regression of tar warts in the rabbit (Rous and Kidd, 1941; MacKenzie and Rous, 1941), and the fact that such tumors could be made to reappear, *often at the identical sites,* by renewed tarring, or even by noncarcinogenic stimuli, such as wound healing or turpentine painting of the previously tarred ears. They drew the important inference from this that "under ordinary circumstances, the tar rendered more cells neoplastic than ever asserted

themselves as visible tumors," and that in warts that had apparently regressed completely, latent tumor cells, irreversibly different from normal cells, could persist for many months, constituting "tumors in a sub-threshold state which require additional aid for progressive neoplasia" (Rous and Kidd, 1941). The concept of initiating and promoting processes, analogous to the two-stage mechanism deduced from the croton oil experiments, was more specifically formulated in a subsequent publication (Friedewald and Rous, 1944).

The term "latent tumor cell," though generally adopted in the literature, is inexact, implying a deficiency in the *neoplastic* quality of the altered cell, instead of merely expressing an inability to manifest itself as a growing tumor mass. The new term "dormant tumor cell" is suggested instead. The term "latent neoplastic potentialities" (Friedewald and Rous, 1950) could then be reserved for the situation in which doubt is felt as to the neoplastic nature of the cells in question.

As further evidence of independent mechanisms for initiating and promoting action is the fact that inhibition by caloric restriction, during continuous carcinogenic painting, is effective in the late stages, but not in the early stages of the latent period of carcinogenesis (Tannenbaum, 1944), though with the carcinogen-croton oil technique, caloric restriction seems to have no influence (Boutwell and Rusch, 1951). A two-stage mechanism also appears to operate with remotely acting carcinogens, e.g., with acetylaminofluorene as initiator and allyl thiourea as promoter, for tumor production in the thyroid (Bielschowsky, 1945; Hall, 1948), or with azo dye as initiator and partial hepatectomy as promoting stimulus, for carcinogenesis in the liver (Glinos *et al.*, 1951). Though one isolated skin tumor arose with croton oil painting preceded by local application of acetylaminofluorene (Kirby, 1948b), none developed when the latter was given by mouth (Ritchie, 1949).

If initiating action converts normal cells into dormant tumor cells, and promoting action causes these dormant tumor cells to develop into visible tumors, it follows that *the number of tumors produced* is determined by the potency of the initiating process, while *the speed with which they appear* (average latent period) is dependent on the efficacy of the promoting process. This was tested by (*a*) varying the carcinogen, or its concentration, for initiating action, and noting the tumor yields following subsequent, standard croton oil treatment, and (*b*) giving a standard dose of carcinogen for initiating action, but varying the time interval between it and the commencement of croton oil treatment, and observing if the average latent period was correspondingly delayed. The anticipated results were quantitatively confirmed (Berenblum and Shubik, 1947b, 1949a,b). The suggestion (Mottram, 1944b) that croton oil produces, in

addition, a "sensitizing" effect prior to initiating action, was not confirmed (Berenblum and Shubik, 1947a). For an attempted mathematical treatment of the two-stage mechanism of carcinogenesis, see Arley and Iversen (1952).

The three most striking features of the initiating process are its specificity, its apparent speed of action (being brought on after a single application of a carcinogen), and its irreversible nature (the anticipated tumor yield being realizable even when the croton oil treatment is delayed for 43 weeks). Since these three features are also characteristic of a gene mutation, it was tempting to consider initiating action as essentially mutational in character. Indeed, the main weakness of the original somatic cell mutation theory of cancer, never adequately stressed by its supporters (Bauer, 1928; Ludford, 1930; Lockhart-Mummery, 1934; Strong, 1949b), was the discrepancy between the remarkably slow evolution of tumor production and the instantaneous nature of a mutation. The two-stage mechanism of carcinogenesis seemed, therefore, to give this theory a new lease of life, by attributing only the *initiating* stage of carcinogenesis to a mutation. When put to the test, by determining whether the powerful mutating agent—sulfur mustard (Auerbach and Robson, 1947)—possessed initiating action on the mouse's skin, the results were negative (Berenblum and Shubik, 1949a). An isolated, negative result may not necessarily disprove a theory. All the same, while many investigators (Auerbach, 1939; Tatum, 1947; Carr, 1948, 1950; Demerec, 1948; Latarjet *et al.*, 1950) have stressed the close correlation between mutagenic and carcinogenic agents, it is clear that the correlation is far from absolute (Latarjet, 1948; Vogt, 1948; Berenblum and Shubik, 1949a; Burdette, 1950). Moreover, the three features of specificity, relative speed of action, and irreversibility, are not necessarily indicative of a mutation; embryonic differentiation being, for instance, an example of a *nonmutational* biological process with these characteristics in common. While the mutation hypothesis remains the most attractive and plausible explanation of initiating action, it cannot yet be said to have been established. The problem is further complicated by recent developments in the concept of *cytoplasmic* genes (plasmagenes) which might also undergo mutations (Haddow, 1944; Darlington, 1948; Holtfreter, 1948). The interesting suggestion has recently been put forward by Danielli (1952) that initiating action of carcinogenesis may be due to a deletion of a chromosome gene and promoting action to a deletion of a plasmagene. This is, however, very speculative.

In contrast to initiating action, promoting action is essentially a gradual process, operative throughout the long latent period of carcinogenesis (see Salaman, 1952), and the effect produced is not strictly

speaking irreversible, seeing that some of the resulting warts have a tendency to regress (see Shubik, 1950b; Friedewald and Rous, 1950). (Many of the induced warts later assume progressive growth, and some even become malignant, but this "progression" probably constitutes a separate and independent process. See below.)

All the known promoting agents are irritants, and as irritation can be defined as "unphysiologic stimulation which, being potentially destructive, elicits a continued state of reparative hyperplasia" (Berenblum, 1944), the simplest explanation for the mechanism of promoting action would be that continued cellular proliferation, of a nonspecific character, was responsible for encouraging the dormant tumor cells to acquire the properties of a growing tumor. This plausible hypothesis became untenable, however, when it was shown (Shubik, 1950a) that many irritants, which were as effective as croton oil in eliciting epithelial hyperplasia, nevertheless failed to function as promoting agents, or else functioned as promoting agents for some species but not for others, despite the fact that cellular proliferation developed in them all. Linnell (1947) found, moreover, that in rabbits, while *deep* skin injuries (punch holes) are effective promoting stimuli, as previously shown by MacKenzie and Rous (1941), damage restricted to the superficial epithelium produces no promoting action, *though the latter is, if anything, the more effective of the two in eliciting epithelial hyperplasia.* Linell (1950) also failed to observe any correlation between the carcinogenic potency of different carcinogens for rabbit skin and the degree of proliferative activity of the epithelium, as tested by eye transplantation. These obseruations not only argued against hyperplasia as a factor, but pointed to the possibility that changes in the subepithelial tissues might be responsible for promoting action.

Such a possibility has often been mooted in the past, before the concept of promoting action was recognized as an independent stage of carcinogenesis. *Hyperemia* has, for instance, been credited with playing a part in carcinogenesis, from histological evidence (Itchikawa and Baum, 1924), or from observations with India ink injections (Kreyberg, 1929; Guldberg, 1931), or from the results of interference with the sympathetic nerve supply (Rémond *et al.*, 1925). Since dilated blood vessels may be evidence of either active hyperemia or passive congestion, while interference with the nerve supply may have many functional effects besides those on the blood vessels, the claim that hyperemia is implicated seems unjustified. Orr (1934), on the contrary, suggested that *ischemia* was involved in the evolution of a tumor, on the basis of the following considerations: (*a*) fibrosis normally develops during the latent period of carcinogenesis (Orr, 1934, 1938; Howes, 1946; Ma, 1949),

causing obliteration of many previously existing or recently formed blood vessels; (*b*) skin carcinogenesis can be augmented by artificially induced fibrosis in the corium (Orr, 1934, 1935); (*c*) in carcinogen-painted skin, a drop in pH (demonstrable by phenol red injection) appears at minute foci where tumors tend subsequently to arise (Orr, 1937); and (*d*) tumor production is augmented by injections of adrenalin or ephedrine sulfate under the painted skin before each application (Orr, 1934, 1935). A surprising feature is that the briefly acting adrenalin was more effective than the more persistently acting ephedrine. (It may be noted, incidentally, that adrenalin is a powerful mitotic inhibitor (Green and Ghadially, 1951).) In applying the technique to the separate stages of carcinogenesis (Ritchie, 1952a), no significant effect of adrenalin was elicited in relation either to the initiating or promoting phase, nor were Orr's original results with adrenalin confirmed. On the other hand, in rabbits, artificially induced ischemia by an uncontroversial method (i.e., by tying off the carotid artery on one side and painting both ears with a carcinogen), led to a definite augmentation of carcinogenesis on the ischaemic side (Ritchie, 1952b).

An interesting new approach to the problem, with the use of transplantation techniques, has provided some encouraging, though inconclusive, support to the view that the derma plays an important role in carcinogenesis. Autologous transplantations of methylcholanthrene-treated skin to normal sites, and of normal skin to sites which had previously been painted with this carcinogen, led to tumor development in the latter, but not in the former (Billingham *et al.*, 1951). The possibility that dormant tumor cells, left behind in the roots of the hair follicles, may have served as sources of tumor growth, and conversely, that the grafts of carcinogen-treated skin may not have taken, are discussed by the authors. (For histological support of the derma playing a role in skin carcinogenesis, see Vernoni, 1952.)

Whether, in the light of these results, ischemia, with or without accompanying fibrosis, should be accepted as a factor favoring carcinogenesis, as part of the actual mechanism of promoting action, still remains an open question.

IX. Histogenesis of Preneoplasia

The clinical definition of a "precancerous" lesion is one in which the probability of a malignant tumor developing is higher than in the equivalent normal tissue. Whether the morphological features of the lesion (hyperplasia, dyskeratosis, fibrosis, etc.) are themselves preneoplastic elements, or whether they are incidental changes accompanying the specific neoplastic process has never been established. Morphological

evidence of clinical material has often been interpreted as supporting the "field effect" hypothesis (Willis, 1948), which postulates that the whole of the hyperplastic zone is somehow implicated in the neoplastic process, though an equally strong case can be made out from morphological studies for the contrary hypothesis of the "single-cell origin of cancer" (Sutton, 1938, 1942).

The problem is no less perplexing in experimentally induced "preneoplasia," for though the morphological changes can here be followed from the outset, with the reasonable assurance that tumors will eventually arise in the precancerous tissue, these tumors, too, are invariably focal in origin, while the preceding hyperplastic changes are diffuse, affecting the whole treated zone. The functional concept of the two-stage mechanism of carcinogenesis, described in the previous section, is at variance with the field effect hypothesis, and presupposes, rather, that preneoplasia consists of isolated dormant tumor cells, lying hidden, throughout the long latent period, among a mass of non-neoplastic cells, *the latter having undergone nonspecific reparative hyperplasia in response to the irritative effects which carcinogens share with noncarcinogenic irritants.*

The distinction between the field effect hypothesis and that of dormant tumor cells, is fundamental, and the implications are far-reaching. It is clear, for instance, that if the dormant tumor cell hypothesis is correct, then metabolic studies of preneoplastic tissues (see Greenstein, 1947; Cowdry, 1947, 1953; Carruthers, 1950) would be of tangential interest only, since the values obtained would merely reflect the nonspecific side effects. Indeed, if a metabolic pattern, believed to be characteristic of tumor tissue, were also found in the stage of preneoplastic hyperplasia, that could be taken as evidence *against* its specificity for neoplasia, since it is inconceivable that the effect of a few single cells would be recognizable in the overall metabolic picture.

The hyperplasia resulting from carcinogenic action has been attributed by Wolbach (1936, 1937) to a secondary (reparative) response to injury, and this has been corroborated for the early changes in the skin (Orr, 1938; Pullinger, 1940; Cramer and Stowell, 1942), subcutaneous tissues (Rondoni, 1937; Orr, 1939), and liver (Orr, 1940; Opie, 1944); though Paletta *et al.* (1941), Pullinger (1940, 1941), Berg (1948), and others, ascribed distinctive features to the preneoplastic hyperplasia of the skin, while Glücksmann (1945) attributed such hyperplastic changes to a primary stimulation of mitotic activity, as distinct from secondary deleterious effects (see below). As for the question of the specificity of preneoplastic changes in the liver, in the early stages of azo dye treatment, this is perhaps too complex to be assessed at present (see Orr, 1940; Opie, 1944, 1947; Price *et al.*, 1952).

The early response of mouse skin to carcinogenic action has been described by many investigators (Orr, 1938; Page, 1938; Pullinger, 1940; Paletta *et al.*, 1941; Glücksmann, 1945; Howes, 1946; Berg, 1948; Ma, 1949; Davibhadhana, 1952; and others). The changes include considerable thickening of the skin, with a matt rather than a shiny surface, and rapid development of alopecia associated with degeneration of the skin appendages, followed by cycles of partial regeneration; hyperplasia of the surface epithelium, with "differentiation" toward a more stratified type; progressive swelling of the epidermal cells, with variations in size of the cells and their nuclei, nuclear distortions and hyperchromatism, and an increase in mitotic and amitotic divisions; a higher nuclear-cytoplasmic ratio, with accentuated prominence of nucleoli; evidence of cytoplasmic degenerations (vacuolation, hyperchromatic staining, perinuclear accumulation of lipoids, etc.), but with a considerable capacity for recovery as indicated by the relative absence of cellular necrosis; changes in the subepithelial tissues, consisting of swelling and fragmentation of collagen fibers and some destruction of elastic fibers, later followed by replacement of fine, nonrefractile, collagen fibrils and (variable) formation of new elastic fibers of somewhat different structure; and dilatation of vascular and lymphatic capillaries, with a sparse accumulation of inflammatory cells.

While none of these changes are, strictly speaking, specific for carcinogenic action, it has been claimed that the speed of their appearance (Orr, 1938; Berg, 1948), and more especially, the relative intensities and proportion of the various features (Pullinger, 1940) differ according to whether the irritant is carcinogenic or not, and if carcinogenic, whether it is potent or weak. While the hyperplasia, and many of the other changes described above, affect the whole painted area of skin, some of the more distinctive features (e.g., variations in nuclear and cell size, and nuclear hyperchromatism) are, according to Paletta *et al.* (1941), focal in distribution, a condition more compatible with the single-cell origin than with the field effect hypothesis.

A very different conclusion, and one more definitely committed to a specific pattern for preneoplastic hyperplasia, was reached by Glücksmann (1945) from differential counts (resting cells, differentiating cells, degenerating cells, and cells in mitosis) in early postnatal and adult mouse skin, and in adult skin treated with barium sulfide, turpentine, benzpyrene, and acetone (solvent control), respectively. The resting cells were increased in absolute amounts, and (less markedly) in relative proportions, in the benzpyrene-treated skin, but not in the barium sulfide-treated skin, while in turpentine-treated skin, the increase was delayed and not as progressive; similarly, the mitotic count was much

increased with benzpyrene, less so with turpentine, and not at all with barium sulfide. Glücksmann concluded that the epidermal thickening was due to cellular migration from the hair follicles, in the case of barium sulfide; to a similar migration, later supplemented by an increased cellular proliferation of the epidermis proper, in the case of turpentine; and to a definite and immediate increase in mitotic activity, *as a primary stimulation of the epidermis,* involving a delay in maturation, rather than a true differentiation, in the case of benzpyrene (and by implication, of carcinogens in general). Applying the same method of analysis to the two-stage mechanism of carcinogenesis, Salaman and Gwynn (1951) claimed that similar differences could be demonstrated as between the action of croton oil on skin pretreated with a carcinogen, and croton oil on normal skin. They concluded that "mouse epidermis, once treated with a chemical carcinogen, though it returns in time to a state almost indistinguishable microscopically from the normal, has suffered a permanent, or at any rate long-lasting, alteration which is general, and does not consist merely in the presence of a few 'latent tumour cells.' "

The validity of these conclusions is naturally determined by the dependability of the method of analysis. By "resting cells" are meant cells that have not yet embarked on the irreversible process of maturation, through various stages of differentiation, towards the ultimate transformation into keratin. The morphological distinction between resting cells and differentiating cells is very slight, and according to the authors themselves (Salaman and Gwynn, 1951), "an observer must to some extent establish his own standard of judgment in distinguishing the different types" (see also Davibhadhana, 1952); the criteria of the transition between resting cell and differentiating cell are based on surmise, not on demonstrable proof; and the differences observed in the various experimental groups are, therefore, semi-quantitative, not qualitative.

Another approach to the problem was from the point of view of the control of the mitotic cycle. Mitotic activity of the mouse skin epidermis is stimulated by estrogens as well as carcinogens (Bullough, 1946), and is inhibited by starvation, or even by a partially restricted diet (Bullough, 1949b), possibly through interference in carbohydrate supply (Bullough, 1949a), though the latter has been questioned by Laws (1952). Its significance, in the present discussion, lies in the fact that dietetic restriction interferes with promoting action (Tannenbaum, 1942a). Bielschowsky and Bullough (1949) observed no interference with tumor induction *when mitotic activity was reduced artificially at the initiating stage.* They did not, unfortunately, investigate the influence of reduced mitotic activity at the promoting stage. (Such an investigation would admittedly involve considerable technical difficulties.)

Mitotic activity *of normal mouse skin* is also inhibited by cortisone (Green and Ghadially, 1951), though not *of skin pretreated with a carcinogen* (Green and Savigear, 1951). However, no difference was found between the effect of cortisone on the hyperplastic response of mouse skin treated with croton oil alone, or treated with croton oil after an initial carcinogenic painting (Ritchie *et al.*, 1953), as might have been expected according to the hypothesis of Salaman and Gwynn (1951).

An interesting application of the principle of a critical size required for the growth of cell colonies, to explain the need of promoting action in carcinogenesis, is supported by a mathematical analysis of the age incidence of cancer in man (Fisher and Hollomon, 1951). The idea merits further study.

X. General Discussion

The two-stage mechanism of carcinogenesis, with the concept of dormant tumor cells being induced by initiating action and converted into growing tumors by promoting action, represents a working hypothesis of tumor pathogenesis, and as such, may serve as a stimulus for further systematic experimentation toward a final solution of the problem. As made clear above, many aspects of the two-stage mechanism are still not understood, and several collateral findings seem, at present, conflicting. Even if ultimately confirmed, and the gaps in our knowledge of it filled in, the hypothesis could never claim to cover the whole range of tumor pathogenesis. The role of tumor viruses is still left out of account (see Rous and Kidd, 1938; Rogers and Rous, 1951); other stages of carcinogenesis, subsequent to promoting action, are also not included in the above scheme.

As regards the latter, some interesting approaches, from at least two different directions, have been made in recent years, which may ultimately have a profound influence on our concept of tumour pathogenesis. Only brief reference can be made here to these other stages, as they are more concerned with the evolution of the tumor than with the initial carcinogenic process.

From a study of the cyclical appearance and growth of spontaneous mammary carcinoma in mice, the appearance and disappearance of tar papillomas in rabbits, the responsiveness of rat fibroadenoma to hormonal influence and their ultimate conversion into carcinoma or sarcoma, and sarcoid transformation of transplanted bladder tumors induced by acetylaminofluorene, Foulds (1941, 1951) postulated the existence of *a reversible stage of "responsiveness"* to extraneous stimuli on the part of benign, and sometimes even of malignant tumors, followed by *a stage of "progression," which represents an irreversible, qualitative change.* (For

analogy to Foulds' "tumors in the responsive state," see Rous and Kidd, 1941; Rogers and Rous, 1950, on conditional growths in rabbit's skin. See also Kline and Rusch, 1944, for progression of induced skin papillomas in mice.) From a study of the behavior of tumors on heterologous transplantation to the anterior chamber of the eye, Greene (1951, 1952) introduced a new concept of *biological autonomy*, which does not necessarily correspond with the clinical or histological concept of malignancy, but bears a much closer relationship to the harmful behavior of certain tumors (e.g., to the tendency to metastasize and the poor survival rate following radical treatment).

The contrast between the classical and the modern ideas about phases of tumor development, represents a decided change in outlook on the whole problem of tumor pathogenesis. In place of the formerly conceived transition from *precancerous hyperplasia to benign tumor to malignant tumor*, new ideas have emerged about *dormant tumor cells*, converted by promoting action to *conditional growths* or *tumors remaining for long in a responsive stage*, but later undergoing an irreversible change into a stage of *progression* or *biological autonomy*.

The simultaneous attack on precancerosis, at one end, and on malignancy, at the other, brings to the problem of tumor pathogenesis a new orientation, which may have important practical implications when some of the missing details are more clearly understood.

REFERENCES

Ahlström, C. G., and Berg, N. O. 1947. *Acta Pathol. Microbiol. Scand.* **24,** 283–298.
Andervont, H. B. 1934. *Public Health Repts. (U.S.)* **49,** 620–624.
Andervont, H. B. 1937. *Public Health Repts. (U.S.)* **52,** 212–221.
Andervont, H. B. 1938. *Public Health Repts. (U.S.)* **53,** 1647–1665.
Andervont, H. B., Grady, H. G., and Edwards, J. E. 1942. *J. Natl. Cancer Inst.* **3,** 131–153.
Andervont, H. B., and Lorenz, E. 1937. *Public Health Repts. (U.S.)* **52,** 637–647.
Andervont, H. B., White, J., and Edwards, J. E. 1944. *J. Natl. Cancer Inst.* **4,** 583–586.
Armstrong, E. C., and Bonser, G. M. 1947. *J. Pathol. Bacteriol.* **59,** 19–27.
Arley, N., and Iversen, S. 1952. *Acta Pathol. Microbiol. Scand.* **31,** 164–171.
Auerbach, C. 1939. *Proc. 7th Intern. Genet. Congr.* pp. 51–52.
Auerbach, C., and Robson, J. M. 1947. *Proc. Roy. Soc. (Edinburgh)* **B62,** 271–283.
Badger, G. M. 1948. *Brit. J. Cancer* **2,** 309–350.
Badger, G. M., Cook, J. W., Hewett, C. L., Kennaway, E. L., Kennaway, N. M., Martin, R. H., and Robinson, A. M. 1940. *Proc. Roy. Soc.* (London) **B129,** 439–467.
Badger, G. M., and Lewis, G. E. 1952. *Brit. J. Cancer* **6,** 270–272.
Barnes, J. M. 1950. *Lancet* **258,** 463.
Barrett, M. K. 1946. *J. Natl. Cancer Inst.* **7,** 127–157.
Bauer, K. H. 1928. Mutationstheorie der Geschwulst-Entstehung. Springer-Verlag, Berlin.

Bauer, K. H. 1949. Das Krebsproblem. Springer-Verlag, Berlin.
Baumann, C. A., and Rusch, H. P. 1939. *Am. J. Cancer* **35**, 213–221.
Berenblum, I. 1929. *J. Pathol. Bacteriol.* **32**, 425–434.
Berenblum, I. 1930. *Brit. J. Exptl. Pathol.* **11**, 208–211.
Berenblum, I. 1931. *J. Pathol. Bacteriol.* **34**, 731–746.
Berenblum, I. 1935. *J. Pathol. Bacteriol.* **40**, 549–558.
Berenblum, I. 1941a. *Cancer Research* **1**, 44–48.
Berenblum, I. 1941b. *Cancer Research* **1**, 807–814.
Berenblum, I. 1944. *Arch. Pathol.* **38**, 233–244.
Berenblum, I. 1945. *Cancer Research* **5**, 561–564.
Berenblum, I. 1949. *J. Natl. Cancer Inst.* **10**, 167–174.
Berenblum, I. 1951a. *J. Natl. Cancer Inst.* **11**, 839–841.
Berenblum, I. 1951b. Symposium on Chemical-Biological Correlation, *Natl. Research Council, Publ. (Washington)* **206**, 363–369.
Berenblum, I. 1952. Clinical Problems in Cancer Research Sloan-Kettering Institute Seminar 1948–1949. 125–143.
Berenblum, I., and Kendal, L. P. 1934. *Brit. J. Exptl. Pathol.* **15**, 366–371.
Berenblum, I., Kendal, L. P., and Orr, J. W. 1936. *Biochem. J.* **30**, 709–715.
Berenblum, I., and Schoental, R. 1942. *Biochem. J.* **36**, 92–97.
Berenblum, I., and Schoental, R. 1943. *Cancer Research* **3**, 145–150.
Berenblum, I., and Schoental, R. 1947a. *Cancer Research* **7**, 390–392.
Berenblum, I., and Schoental, R. 1947b. *Brit. J. Cancer* **1**, 157–165.
Berenblum, I., and Shubik, P. 1947a. *Brit. J. Cancer* **1**, 379–382.
Berenblum, I., and Shubik, P. 1947b. *Brit. J. Cancer* **1**, 383–391.
Berenblum, I., and Shubik, P. 1949a. *Brit. J. Cancer* **3**, 109–118.
Berenblum, I., and Shubik, P. 1949b. *Brit. J. Cancer* **3**, 384–386.
Berg, N. O. 1948. *Acta Pathol. Microbiol. Scand.* **25**, 34–44.
Bernelli-Zazzera, A. 1952. *Tumori* **38**, 339–350.
Berrill, N. J. 1943. *Physiol. Revs.* **23**, 101–123.
Bielschowsky, F. 1944. *Brit. J. Exptl. Pathol.* **25**, 1–4.
Bielschowsky, F. 1945. *Brit. J. Exptl. Pathol.* **26**, 270–275.
Bielschowsky, F., and Bullough, W. S. 1949. *Brit. J. Cancer* **3**, 282–285.
Bielschowsky, F., and Griesbach, W. E. 1950. *Brit. J. Cancer* **4**, 133–138.
Billingham, R. E., Orr, J. W., and Woodhouse, D. L. 1951. *Brit. J. Cancer* **5**, 417–432.
Biskind, M. S., and Biskind, G. R. 1944. *Proc. Soc. Exptl. Biol. Med.* **55**, 176–179.
Biskind, M. S., and Biskind, G. R. 1945. *Proc. Soc. Exptl. Biol. Med.* **59**, 4–8.
Bittner, J. J. 1942. *Cancer Research* **2**, 710–721.
Blum, H. F. 1948. *J. Natl. Cancer Inst.* **9**, 245–258.
Blum, H. F. 1950. *J. Natl. Cancer Inst.* **11**, 463–495.
Bonne, C., Lodder, J., and Streef, G. M. 1930. *Z. Krebsforsch.* **32**, 310–326.
Bonser, G. M. 1938. *J. Pathol. Bacteriol.* **46**, 581–602.
Bonser, G. M 1940. *Am. J. Cancer* **38**, 319–327.
Bonser, G. M., Clayson, D. B., and Jull, J. W. 1951. *Lancet* **261**, 286–288.
Bonser, G. M., Clayson, D. B., Jull, J. W., and Pyrah, L. N. 1952. *Brit. J. Cancer* **6**, 412–424.
Bonser, G. M., and Green, H. N. 1950. *J. Pathol. Bacteriol.* **62**, 531–539.
Bonser, G. M., and Jull, J. W. 1952. 30th Ann. Rept. Brit. Emp. Cancer Campaign. p. 180.
Bonser, G. M., and Robson, J. M. 1940. *J. Pathol. Bacteriol.* **51**, 9–21.
Boutwell, R. K., and Rusch, H. P. 1951. *Cancer Research* **11**, 238–239.

Boyland, E. 1950. *Biochem. Soc. Symposia* (*Cambridge Engl.*) No. 5, 40–54.
Boyland, E. 1952. *Cancer Research.* **12,** 77–84.
Boyland, E., and Burrows, H. 1935. *J. Path. Bact.* **41,** 231–238.
Boyland, E., and Horning, E. S. 1949. *Brit. J. Cancer* **3,** 118–123.
Boyland, E., Levi, A. A., Mawson, E. H., and Roe, E. 1941. *Biochem. J.* **35,** 184–191.
Boyland, E., and Warren, F. L. 1937. *J. Pathol. Bacteriol.* **45,** 171–177.
Boyland, E., and Weigert, F. 1947. *Brit. Med. Bull.* **4,** 354–359.
Browning, C. H., Gulbransen, R., and Niven, J. S. F. 1936. *J. Pathol. Bacteriol.* **42,** 155–159.
Brunschwig, A., and Bissell, A. D. 1938. *Arch. Surg.* **36,** 53–60.
Brunschwig, A., and Tschetter, D. 1937. *Proc. Soc. Exptl. Biol. Med.* **36,** 439–441.
Brunschwig, A., Tschetter, D., and Bissell, A. D. 1937. *Ann. Surg.* **106,** 1084–1088.
Bryan, W. R., and Shimkin, M. B. 1943. *J. Natl. Cancer Inst.* **3,** 503–531.
Bullough, W. S. 1946. *Phil. Trans. Roy. Soc.* (*London*) **B231,** 453–516.
Bullough, W. S. 1949a. *J. Exptl. Biol.* **26,** 83–99.
Bullough, W. S. 1949b. *Brit. J. Cancer* **3,** 275–282.
Burdette, W. J. 1950. *Science* **112,** 303–306.
Burdette, W. J., and Strong, L. C. 1943. *Cancer Research* **3,** 13–20.
Burk, D., and Winzler, R. J. 1944. *Vitamins and Hormones* **2,** 305–352.
Burrows, H. 1932. *Proc. Roy. Soc.* (*London*) **B111,** 238–247.
Burrows, H. 1933. *Am. J. Cancer* **17,** 1–6.
Burrows, H., Hieger, I., and Kennaway, E. L. 1932. *Am. J. Cancer* **16,** 57–67.
Burrows, H., Hieger, I., and Kennaway, E. L. 1936. *J. Pathol. Bacteriol.* **43,** 419–426.
Burrows, H., and Horning, E. 1952. Oestrogens and Neoplasia. Blackwell Sci. Publ. Oxford.
Burrows, H., Mayneord, W. V., and Roberts, J. E. 1937. *Proc. Roy. Soc.* (*London*) **B123,** 213–217.
Cabot, S., Shear, M. J., and Perrault, A. 1940. *Am. J. Pathol.* **16,** 301–312.
Cantarow, A., Paschkis, K. E., Stasney, J., and Rothenberg, M. S. 1946. *Cancer Research* **6,** 610–616.
Cantarow, A., Stasney, J., and Paschkis, K. E. 1948. *Cancer Research* **8,** 412–417.
Calcutt, G. 1949. *Brit. J. Cancer* **3,** 306–310.
Carr, J. G. 1948. *Brit. J. Cancer* **2,** 132–134.
Carr, J. G. 1950. *Biochem. Soc. Symposia* (*Cambridge Engl.*) No. 4, 25–31.
Carruthers, C. 1940. *Arch. Pathol.* **30,** 1184–1191.
Carruthers, C. 1950. *Cancer Research* **10,** 255–265.
Chevallier, A., Manuel, S., and Denoix, P. 1946. *Bull. assoc. franç. étude cancer* **33,** 223–238.
Choldin, S. 1927. *Z. Krebsforsch.* **25,** 235–246.
Choldin, S. 1930. *Z. Krebsforsch.* **31,** 545–556.
Cook, J. W. 1948. *Brit. J. Nutrition* **1,** 245–253.
Cook, J. W., Duffy, E., and Schoental, R. 1950. *Brit. J. Cancer* **4,** 405–410.
Cook, J. W., Haslewood, G. A. D., Hewett, C. L., Hieger, I., Kennaway, E. L., and Mayneord, W. V. 1937. *Am. J. Cancer* **29,** 219–257.
Cook, J. W., and Kennaway, E. L. 1938. *Am. J. Cancer* **33,** 50–97.
Cook, J. W., and Kennaway, E. L. 1940. *Am. J. Cancer* **39,** 381–428 and 521–582.
Cook, J. W., and Schoental, R. 1952. *Brit. J. Cancer* **8,** 400–406.
Copeland, D. H., and Salmon, W. D. 1946. *Am. J. Pathol.* **22,** 1059–1079.
Coulson, C. A. 1953. *Advances in Cancer Research,* **1,** 1–56.
Cowdry, E. V. 1947. *J. Am. Med. Assoc.* **135,** 408–411.

Cowdry, E. V. 1953. *Advances in Cancer Research* **1**, 57–101.
Cowdry, E. V., and Suntzeff, V. 1944. *Yale J. Biol. Med.* **17**, 47–58.
Crabtree, H. G. 1940a. *J. Pathol. Bacteriol.* **51**, 299–302.
Crabtree, H. G. 1940b. *J. Pathol. Bacteriol.* **51**, 303–309.
Crabtree, H. G. 1941a. *Cancer Research* **1**, 34–38.
Crabtree, H. G. 1941b. *Cancer Research* **1**, 39–43.
Crabtree, H. G. 1948. *Brit. J. Cancer* **2**, 281–289.
Cramer, W., and Horning, E. S. 1936. *Lancet* **247**, 1056–1057.
Cramer, W., and Stowell, R. E. 1942. *J. Natl. Cancer Inst.* **2**, 379–402.
Cramer, W., and Stowell, R. E. 1943. *Cancer Research* **3**, 36–42.
Danielli, J. F. 1952. *Nature* **170**, 1042–1044.
Darlington, C. D. 1948. *Brit. J. Cancer* **2**, 118–126.
Daudel, P., and Daudel, R. 1950. *Biol. Med.*, **39**, 201–236.
Davibhadhana, B. 1952. *Cancer Research* **12**, 165–169.
Deelman, H. T. 1923. *Bull. Assoc. franç. étude cancer* **12**, 24–30.
Deelman, H. T., and Van Erp, J. P. 1926. *Z. Krebsforsch.* **24**, 86–98.
Demerec, M. 1948. *Brit. J. Cancer* **2**, 114–117.
Derom, E. 1924. *Bull. Assoc. franç. étude cancer* **13**, 422–462.
Des Ligneris, M. J. A. 1940. *Am. J. Cancer* **40**, 1–46.
Dickens, F. 1947. *Brit. Med. Bull.* **4**, 348–354.
Dickens, F., and Weil-Malherbe, H. 1942. *Cancer Research* **2**, 560–566.
Dickens, F., and Weil-Malherbe, H. 1946. *Cancer Research* **6**, 161–170.
Dmochowski, L., and Orr, J. W. 1949. *Brit. J. Cancer* **3**, 376–384.
Doniach, I. 1950. *Brit. J. Cancer* **4**, 223–234.
Doniach, I., and Mottram, J. C. 1940. *Am. J. Cancer* **39**, 234–240.
Dormans, E. 1934. *Z. Krebsforsch.* **40**, 577–584.
Dunning, W. F., Curtis, M. R., and Bullock, F. D. 1936. *Am. J. Cancer* **28**, 681–712.
Dunning, W. F., Curtis, M. R., and Madson, M. E. 1947. *Cancer Research* **7**, 134–140.
Dunning, W. F., Curtis, M. R., and Wood, F. C. 1940. *Am. J. Cancer* **39**, 70–93.
Duran-Reynals, F., Shrigley, E. W., and de Hostos, E. 1945. *Cancer Research* **5**, 11–17.
du Vigneaud, V., Spangler J. M., Burk, D., Kensler, D. C., Sugiura, K., and Rhoads, C. P. 1942. *Science* **95**, 174–176.
Edwards, J. E. 1941. *J. Natl. Cancer Inst.* **2**, 197–199.
Ekwall, P., Ermala, P., Setälä, K., and Sjöblom, L. 1951. *Cancer Research* **11**, 758–763.
Ekwall, P., and Setälä, K. 1948. *Acta Chem. Scand.* **2**, 733–739.
Ekwall, P., and Setälä, K. 1950. *Acta unio internat. contra Cancrum* **7**, 120–125.
Engel, R. W., and Copeland, D. H. 1952. *Cancer Research* **12**, 211–215.
Engelbreth-Holm, J. 1941. *Cancer Research* **1**, 109–112.
Esmarch, O. 1942. *Acta Pathol. Microbiol. Scand.* **19**, 79–99.
Fieser, L. F. 1938. *Am. J. Cancer* **34**, 37–124.
Fieser, L. F. 1940. *In* Cause and Growth of Cancer, pp. 1–27. Univ. Pennsylvania Bicent. Conf.
Findlay, G. M. 1928. *Lancet* **215**, 1070–1073.
Fisher, J. C., and Hollomon, J. H. 1951. *Cancer* **4**, 916–918.
Fitzhugh, O. G., and Nelson, A. A. 1948. *Science* **108**, 626–628.
Foulds, L. 1947. *Brit. J. Cancer* **1**, 172–176.
Foulds, L. 1949. *Brit. J. Cancer* **3**, 345–375.
Foulds, L. 1951. *Ann. Roy. Coll. Surg.* **9**, 93–101.
Friedewald, W. F., and Rous, P. 1944a. *J. Exptl. Med.* **80**, 101–126.
Friedewald, W. F., and Rous, P. 1944b. *J. Exptl. Med.* **80**, 127–144.

Friedewald, W. F., and Rous, P. 1950. *J. Exptl. Med.* **91,** 459–484.
Furth, J., and Furth, O. B. 1936. *Am. J. Cancer* **28,** 54–65.
Gardner, L. U., and Heslington, H. F. 1946. *Federation Proc.* **5,** 221.
Gardner, W. U. 1947. *In* Endocrinology of Neoplastic diseases, pp. 11–44. Oxford University Press.
Gardner, W. U. 1948. *Cancer Research* **8,** 397–411.
Gardner, W. U. 1953. *Advances in Cancer Research* **1,** 173–232.
Gardner, W. U., Smith, G. M., Strong, L. C., and Allen, E. 1936. *Arch. Pathol.* **21,** 504–508.
Gilmour, M. D. 1937. *J. Path. Bact.* **45,** 179–188.
Glinos, A. D., Bucher, N. L. R., and Aub, J. C. 1951. *J. Exptl. Med.* **93,** 313–324.
Glücksmann, A. 1945. *Cancer Research* **5,** 385–400.
Goldberg, R. C., and Chaikoff, I. L. 1952. *Arch. Pathol.* **53,** 22–28.
Gordonoff, T., and Ludwig, F. 1938. *Z. Krebsforsch.* **47,** 421–426.
Graffi, A. 1939. *Z. Krebsforsch.* **49,** 477–495.
Green, H. N., and Ghadially, F. N. 1951. *Brit. Med. J. i,* 496–498.
Green, H. N., and Savigear, M. 1951. *Brit. Med. J.,* **1,** 498–500.
Greene, H. S. N. 1945. *Science* **101,** 644–645.
Greene, H. S. N. 1951. *Cancer Research* **11,** 899–903.
Greene, H. S. N. 1952. *Cancer* **5,** 24–44.
Greenstein, J. P. 1947. Biochemistry of Cancer. Academic Press, New York.
Griffin, A. C., Brandt, E. L., and Setter, V. 1951. *Cancer Research* **11,** 868–872.
Griffin, A. C., Brandt, E. L., and Tatum, E. L. 1950. *J. Am. Med. Assoc.* **144,** 571.
Griffin, A. C., Rinfret, A. P., and Corsigilia, V. F. 1953. *Cancer Research* **13,** 77–79.
Guldberg, G. 1931. *Acta Pathol. Microbiol. Scand. Suppl.* **8,** 1–223.
Gwynn, R. H., and Salaman, M. H. 1951. 29th Ann. Rept., Brit. Emp. Cancer Campaign, p. 115.
Haagensen, C. D., and Krehbiel, O. F. 1936. *Am. J. Cancer* **26,** 368–377.
Haddow, A. 1944. *Nature* **154,** 194–199.
Hall, G. E., and Franks, W. R. 1938. *J. Biol. Chem.* **123,** 49.
Hall, W. H. 1948. *Brit. J. Cancer* **2,** 273–280.
Hare, W. V., Stewart, H. L., Bennett, J. G., and Lorenz, E. 1952. *J. Natl. Cancer Inst.* **12,** 1019–1055.
Harris, P. N., and Clowes, G. H. A. 1952. *Cancer Research* **12,** 471–479.
Hartwell, J. L. 1951. Survey of Compounds Which Have Been Tested for Carcinogenic Activity, 2nd edition. Public Health Service Publ. No. 149. Federal Security Agency, Washington, D.C.
Heidelberger, C., and Weiss, S. M. 1951. *Cancer Research* **11,** 885–891.
Heidelberger, C., and Wiest, W. G. 1951. *Cancer Research* **11,** 511–518.
Hendry, J. A., Homer, R. F., and Rose, F. L. 1951. *Brit. J. Pharmacol.* **6,** 235–255.
Henshaw, P. S. 1945. *J. Natl. Cancer Inst.* **5,** 419–436.
Henshaw, P. S., and Meyer, H. L. 1944. *J. Natl. Cancer Inst.* **4,** 523–525.
Hertz, R. 1951. *Cancer Research* **11,** 393–397.
Heston, W. E. 1940. *J. Natl. Cancer Inst.* **1,** 105–111.
Heston, W. E. 1950. *J. Natl. Cancer Inst.* **11,** 415–423.
Heston, W. E., and Dunn, T. B. 1951. *J. Natl. Cancer Inst.* **11,** 1057–1071.
Hieger, I. 1936. *Am. J. Cancer* **28,** 522–529.
Hill, W. T., Stanger, D. W., Pizzo, A., Riegel, B., Shubik, P., and Wartman, W. B. 1951. *Cancer Research* **11,** 892–897.

Hill, W. T., Stanger, D. W., Pizzo, A., Riegel, B., and Wartman, W. B. 1952. *Cancer Research* **12**, 270–271.
Hitchcock, C. R. 1952. *J. Natl. Cancer Inst.* **12**, 723–733.
Hoch-Ligetti, C. 1949. *Brit. J. Cancer* **3**, 285–288.
Holtfreter, J. 1948. *Symposia Soc. Exptl. Biol.* **2**, 17–49.
Hooker, C. W., Gardner, W. U., and Pfeiffer, C. A. 1940. *J. Am. Med. Assoc.* **115**, 443–445.
Horning, E. S. 1946. *Lancet* **251**, 829–830.
Horning, E. S. 1947. *Lancet* **253**, 207–209.
Horning, E. S. 1949. *Brit. J. Cancer* **3**, 211–230.
Howes, E. L. 1946. *Cancer Research* **6**, 298–310.
Hueper, W. C. 1942. Occupational Tumors and Allied Diseases. Charles C Thomas, Springfield, Ill.
Hueper, W. C. 1951. *Texas Rept. Biol. Med.* **10**, 167–186.
Hueper, W. C., Wiley, F. H., and Wolfe, H. D. 1938. *J. Indust. Hyg. & Toxicol.* **20**, 46–84.
Hueper, W. C., Zuefle, J. H., Link, A. M., and Johnson, M. G. 1952. *J. Natl. Cancer Inst.* **13**, 291–301.
Iball, J. 1939. *Am. J. Cancer* **35**, 188–190.
Ilfeld, F. W. 1936. *Am. J. Cancer* **26**, 743–753.
Itchikawa, K., and Baum, S. M. 1924. *Bull. Assoc. franç. étude cancer* **13**, 386–395.
Jacobi, H. P., and Baumann, C. A. 1940. *Am. J. Cancer* **39**, 338–342.
Jaffé, W. G. 1947a. *Cancer Research* **7**, 107–112.
Jaffé, W. G. 1947b. *Cancer Research* **7**, 113–116.
Jonkhoff, A. E. 1927. *Z. Krebsforsch.* **26**, 32–41.
Jull, J. W. 1951. *Brit. J. Cancer* **5**, 328–330.
Kensler, C. J. 1949. *J. Biol. Chem.* **179**, 1079–1084.
Kensler, C. J. 1952. Texas Univ. Monogr. (In press).
Kensler, C. J., Sugiura, K., Young, N. F., Halter, C. R., and Rhoads, C. P. 1941. *Science* **93**, 308–310.
Kinosita, R. 1937. *Trans. Japan. Pathol. Soc.* **27**, 665–725.
Kirby, A. H. M. 1948a. *Brit. J. Cancer* **2**, 290–294.
Kirby, A. H. M. 1948b. *Brit. J. Cancer* **2**, 294–300.
Kirkman, H. 1952. *Cancer Research* **12**, 274–275.
Kirschbaum, A. 1951. *Cancer Research* **11**, 741–752.
Kirschbaum, A., Williams, W. L., and Bittner, J. J. 1946. *Cancer Research* **6**, 354–362.
Klein, A. J., and Palmer, W. L. 1940. *Arch. Pathol.* **29**, 814–844.
Klein, M. 1951. *J. Natl. Cancer Inst.* **11**, 843–848.
Klein, M. 1952. *J. Natl. Cancer Inst.* **12**, 735–742.
Kline, B. E., and Rusch, H. P. 1944. *Cancer Research* **4**, 762–767.
Kohn-Speyer, A. C. 1929. *Lancet* **217**, 1305–1306.
Korpássy, B., and Mosonyi, M. 1950. *Brit. J. Cancer* **4**, 411–420.
Korteweg, R., and Thomas, F. 1939. *Am. J. Cancer* **37**, 36–44.
Krebs, C. 1928. *Z. Immunitätsforsch.* **59**, 203–218.
Kreyberg, L. 1929. *Virchow's Arch. pathol. Anat. u. Physiol.* **273**, 367–440.
Kreyberg, L. 1935. *Am. J. Cancer* **24**, 554–565.
Lacassagne, A. 1932. *Compt. rend. Acad. Sc.* **195**, 630–632.
Lacassagne, A. 1933. *Compt. rend. Acad. Sc.* **196**, 69–71.
Lacassagne, A. 1936. *Am. J. Cancer* **28**, 735–740.
Lacassagne, A. 1937. *Compt. rend. soc. biol.* **126**, 193–195.

Lacassagne, A. 1938a. *Bull. Assoc. franç. étude cancer* **27,** 96–116.
Lacassagne, A. 1938b. *Compt. rend. soc. biol.* **129,** 641–643.
Lacassagne, A. 1946. Les Cancers Produit par des Substances Chimiques Exogènes. Hermann et Cie., Paris.
Lacassagne, A. 1947. Etude de la Cancérisation par les Substances Chimiques Exogènes. Hermann et Cie., Paris.
Lacassagne, A., Buü Hoi, N. P., and Rudali, G. 1945. *Brit. J. Exptl. Pathol.* **26,** 5–12.
Larionov, L. F. 1930. Voprosy Onkologii, Kharkov **3,** 114; (abstr. 1931. *Am. J. Cancer* **15,** 2832).
Larsen, C. D., and Heston, W. E. 1945. *J. Natl. Cancer Inst.* **6,** 31–40.
Latarjet, R. 1948. *Compt. rend. soc. biol.* **142,** 453.
Latarjet, R., Buü-Hoi, N. P. and Elias, C. A. 1950. *Pubbl. staz. zool. Napoli, Suppl.* **22,** 78–93.
Lathrop, A. E. C., and Loeb, L. 1916. *J. Cancer Research* **1,** 1–20.
Lauridsen, J., and Eggers, H. E. 1943. *Cancer Research* **3,** 43–46.
Lavik, P. S., and Baumann, C. A. 1941. *Cancer Research* **1,** 181–187.
Lavik, P. S., Moore, P. R., Rusch, H. P., and Baumann, C. A. 1942. *Cancer Research* **2,** 189–192.
Law, L. W. 1941a. *Cancer Research* **1,** 397–401.
Law, L. W. 1941b. *Cancer Research* **1,** 564–571.
Law, L. W. 1941c. *Am. J. Pathol.* **17,** 827–831.
Laws, J. O. 1952. *J. Exptl. Biol.* **29,** 328–335.
Leathem, J. H. 1951. *Cancer Research* **11,** 266.
Lefèvre, H. 1945. Acceleration of the Development of Spontaneous Tumours in Mice. Thaning & Appels Forlag., Copenhagen.
Leitch, A., and Kennaway, E. L. 1922. *Brit. Med. J. ii,* 451.
Leiter, J., and Shear, M. J. 1943. *J. Natl. Cancer Inst.* **3,** 455–477.
Li, M. H., and Gardner, W. U. 1947. *Cancer Research* **7,** 549–566.
Linell, F. 1947. *Acta Pathol. Microbiol. Scand. Suppl.* **71.**
Linell, F. 1950. *Acta Pathol. Microbiol. Scand.* **27,** 662–670.
Lipschutz, A. 1950. Steroid Hormones and Tumors. Williams & Wilkins Co., Baltimore.
Lipschutz, A. 1951. *Rev. can. biol.* **10,** 341–383.
Lipschutz, A., and Iglesias, R. 1938. *Compt. rend. soc. biol.* **129,** 519–524.
Lisco, H., Finkel, M. P., and Brues, A. M. 1947. *Radiology* **49,** 361–362.
Little, C. C. 1947. *Biol. Revs.* **22,** 315–343.
Loeb, L. 1940. *J. Natl. Cancer Inst.* **1,** 169–196.
Loeb, L. 1947. *Bull. N.Y. Acad. Med.* **23,** 564–580.
Lorenz, E., Eschenbrenner, A. B., Heston, W. E., and Uphoff, D. 1951. *J. Natl. Cancer Inst.* **11,** 947–965.
Lorenz, E., and Stewart, H. L. 1940. *J. Natl. Cancer Inst.* **1,** 17–40.
Lockhart-Mummery, J. P. 1934. The Origin of Cancer. J. & A. Churchill, London.
Ludford, R. J. 1929. *Brit. J. Exptl. Pathol.* **10,** 193–196.
Ludford, R. J. 1930. *9th Rept. Imperial Cancer Research Fund* 121–147.
Ma, C. K. 1949. *Cancer Research* **9,** 481–487.
MacDonald, J. C., Miller, E. C., Miller, J. A., and Rusch, H. P. 1952. *Cancer Research* **12,** 50–54.
MacKenzie, I., and Rous, P. 1941. *J. Exptl. Med.* **73,** 391–416.
McEuen, C. S. 1939. *Am. J. Cancer* **36,** 551–566.
Maisin, J. 1948. Cancer. Casterman, Tournai-Paris.
Maisin, J., and Coolen, M. L. 1936. *Compt. rend. soc. biol.* **123,** 159–160.

Marie, P., Clunet, J., and Raulot-Lapointe, G. 1910. *Bull. Assoc. franç. étude cancer.* **3,** 404–426.
Medawar, P. B. 1947. *Biol. Revs.* **22,** 360–389.
Meyenburg, H. v., and Fritzsche, H. 1943. *Schweiz. med. Wochschr.* **73,** 201–204.
Michalowsky, I. 1928. *Virchow's Arch. pathol. Anat. u. Physiol.* **267,** 27–62.
Mider, G. B., and Morton, J. J. 1939. *Am. J. Pathol.* **15,** 299–302.
Miller, J. A. 1947. *Ann. N.Y. Acad. Sci.* **49,** 19–28.
Miller, J. A., and Miller, E. C. 1948. *J. Exptl. Med.* **87,** 139–156.
Miller, E. C., Miller, J. A., and Brown, R. R. 1952. *Cancer Research* **12,** 282–283.
Miller, J. A., and Miller, E. C. 1953. *Advances in Cancer Research* **1,** 339–396.
Miner, D. L., Miller, J. A., Baumann, C. A., and Rusch, H. P. 1943. *Cancer Research* **3,** 296–302.
Moon, H. D., Simpson, M. E., and Evans, H. M. 1952. *Science* **116,** 331.
Moon, H. D., Simpson, M. E., Li, C. M. L., and Evans, H. M. 1950a. *Cancer Research* **10,** 297–308.
Moon, H. D., Simpson, M. E., Li, C. M. L., and Evans, H. M. 1950b. *Cancer Research* **10,** 364–370.
Moon, H. D., Simpson, M. E., Li, C. M. L., and Evans, H. M. 1950c. *Cancer Research* **10,** 549–556.
Moon, H. D., Simpson, M. E., Li, C. M. L., and Evans, H. M. 1951. *Cancer Research* **11,** 535–537.
Moore, R. A., and Melchionna, R. H. 1937. *Am. J. Cancer* **30,** 731–741.
Morris, H. P. 1952. *Texas Rept. Biol. Med.* **10,** 1028–1054.
Morton, J. J., and Mider, G. B. 1938. *Science* **87,** 327–328.
Morton, J. J., and Mider, G. B. 1939. *Proc. Soc. Exptl. Biol. Med.* **41,** 357–360.
Mottram, J. C. 1937. *Am. J. Cancer* **30,** 746–748.
Mottram, J. C. 1938. *Am. J. Cancer* **32,** 76–79.
Mottram, J. C. 1944a. *J. Pathol. Bacteriol.* **56,** 181–187.
Mottram, J. C. 1944b. *J. Pathol. Bacteriol.* **56,** 391–402.
Mueller, G. J., and Miller, J. A. 1950. *J. Biol. Chem.* **185,** 145–154.
Narat, J. K. 1925. *J. Cancer Research* **9,** 135–147.
Nelson, A. A., Fitzhugh, O. G., and Calvery, H. O. 1943. *Cancer Research* **3,** 230–236.
Nettleship, A., and Henshaw, P. H. 1943. *J. Natl. Cancer Inst.* **4,** 309–319.
Oberling, C. 1944. The Riddle of Cancer. Yale University Press., New Haven.
Oberling, C., and Guérin, M. 1947. *Bull. Assoc. franç. étude Cancer* **34,** 30–42.
Oberling, C., Guérin, M., and Guérin, P. 1939a. *Bull. Assoc. franç. étude Cancer* **28,** 198–213.
Oberling, C., Guérin, P., Guérin, M., and Sannié, C. 1939b. *Compt. rend. soc. biol.* **130,** 17–19.
Oberling, C., Sannié, C., Guérin, M., and Guérin, P. 1936. *Bull. Assoc. franç. étude Cancer* **25,** 156–180.
Opie, E. L. 1944. *J. Exptl. Med.* **80,** 219–230.
Opie, E. L. 1947. *J. Exptl. Med.* **86,** 45–54.
Oppenheimer, B. S., Oppenheimer, E. T., and Stout, A. P. 1948. *Trans. Assoc. Am. Physicians* **61,** 343–348.
Orr, J. W. 1934. *Brit. J. Exptl. Pathol.* **15,** 73–79.
Orr, J. W. 1935. *Brit. J. Exptl. Pathol.* **16,** 121–126.
Orr, J. W. 1937. *J. Pathol. Bacteriol.* **44,** 19–27.
Orr, J. W. 1938. *J. Pathol. Bacteriol.* **46,** 495–515.
Orr, J. W. 1939. *J. Pathol. Bacteriol.* **49,** 157–170.

Orr, J. W. 1940. *J. Pathol. Bacteriol.* **50,** 393–408.
Orr, J. W. 1947. *Brit. Med. Bull.* **4,** 385–388.
Page, R. C. 1938. *Arch. Pathol.* **26,** 800–813.
Paletta, F. X., Cowdry, E. V., and Lischer, C. E. 1941. *Cancer Research* **1,** 942–952.
Paletta, F. X., and Max, P. F. 1942. *J. Natl. Cancer Inst.* **2,** 577–581.
Paschkis, K. E., Cantarow, A., and Stasney, J. 1948. *Cancer Research* **8,** 257–263.
Passey, R. D. 1938. *J. Pathol. Bacteriol.* **47,** 349–351.
Peacock, P. R. 1933. *J. Pathol. Bacteriol.* **36,** 141–152.
Peacock, P. R. 1935. *Proc. Leeuwenhoek Vereeninging* (cited by Dickens, 1947).
Peacock, P. R. 1936. *Brit. J. Exptl. Pathol.* **17,** 164–172.
Peacock, P. R., and Beck, S. 1938. *Brit. J. Exptl. Pathol.* **19,** 315–319.
Peacock, P. R., Beck, S., and Anderson, W. 1949. *Brit. J. Cancer* **3,** 296–305.
Pott, P. 1775. Chirurgical Observations, etc., London 61–68 (cited by Haagensen, C. D. 1931. *Am. J. Cancer* **15,** 641–703).
Price, J. M., Harman, J. W., Miller, E. C., and Miller, J. A. 1952. *Cancer Research* **12,** 192–200.
Pullinger, B. D. 1940. *J. Pathol. Bacteriol.* **50,** 463–471.
Pullinger, B. D. 1941. *J. Pathol. Bacteriol.* **53,** 287–288.
Pullinger, B. D. 1943. *J. Pathol. Bacteriol.* **55,** 301–309.
Pullinger, B. D. 1945. *J. Pathol. Bacteriol.* **57,** 477–481.
Purves, H. D., and Griesbach, W. E. 1947. *Brit. J. Exptl. Pathol.* **28,** 46–53.
Raposo, L. S. 1928. *Compt. rend. soc. biol.* **98,** 999–1001.
Rask-Nielsen, R. 1948. *Acta Pathol. Microbiol. Scand. Suppl.* **78,**
Rask-Nielsen, R. 1950a. *Brit. J. Cancer* **4,** 117–123.
Rask-Nielsen, R. 1950b. *Brit. J. Cancer* **4,** 124–132.
Reimann, S. P., and Hall, E. M. 1936. *Arch. Pathol.* **22,** 55–61.
Rémond, A., Bernardbeig, J., and Sendrail, M. 1925. *Compt. rend. soc. biol.* **93,** 1063.
Riegel, B., Wartman, W. B., Hill, W. T., Reeb, B. B., Shubik, P., and Stanger, D. W. 1951. *Cancer Research* **11,** 301–303.
Richardson, H. L., and Cunningham, L. 1951. *Cancer Research* **11,** 274.
Riley, J. F., and Pettigrew, F. W. 1945. *Brit. J. Exptl. Pathol.* **26,** 63–66.
Ritchie, A. C. 1949. 27th Ann. Rept. Brit. Emp. Cancer Campaign, p. 203.
Ritchie, A. C. 1952a. *J. Natl. Cancer Inst.* **12,** 839–846.
Ritchie, A. C. 1952b. *J. Natl. Cancer Inst.* **12,** 847–850.
Ritchie, A. C., Shubik, P., Lane, M., and Leroy, E. P. 1953. *Cancer Research* **13,** 45–48.
Rogers, S. 1951. *Cancer Research* **11,** 275–276.
Rogers, S., and Rous, P. 1951. *J. Exptl. Med.* **93,** 459–488.
Rondoni, P. 1937. *Z. Krebsforsch.* **47,** 59–83.
Rondoni, P. 1946. Il Cancro. Milano.
Rondoni, P., and Corbellini, A. 1936. *Tumori* **10,** 106–118.
Rosicki, J., and Hatschek, R. 1943. *Z. Krebsforsch.* **54,** 26–38; (Abstr. *Cancer Research* 1945. **5,** 120).
Ross, J. M. 1936. *J. Pathol. Bacteriol.* **43,** 267–276.
Rous, P., and Kidd, J. G. 1938. *J. Exptl. Med.* **67,** 399–428.
Rous, P., and Kidd, J. G. 1941. *J. Exptl. Med.* **73,** 365–390.
Rous, P., and Smith, W. E. 1945. *J. Exptl. Med.* **81,** 597–620.
Roussy, G., Leroux, R., and Peyre, E. 1924. *Bull. Assoc. franç. étude Cancer* **13,** 587–589.
Rumsfeld, H. W., Miller, W. L., and Baumann, C. A. 1951. *Cancer Research* **11,** 814–819.

Rusch, H. P. 1944. *Physiol. Revs.* **24**, 177–204.
Rusch, H. P., Baumann, C. A., Miller, J. A., and Kline, B. E. 1945a. Research Conf. on Cancer. *Publ. Am. Assoc. Advance Sci.* pp. 267–286.
Rusch, H. P., Johnson, R. O., and Kline, B. E. 1945b. *Cancer Research* **5**, 705–712.
Rusch, H. P., Kline, B. E., and Baumann, C. A. 1941. *Arch. Pathol.* **31**, 135–146.
Rusch, H. P., Kline, B. E., and Baumann, C. A. 1942. *Cancer Research* **2**, 183–188.
Rusch, H. P., Kline, B. E., and Baumann, C. A. 1945c. *Cancer Research* **5**, 431–435.
Russell, W. O., and Ortega, L. R. 1952. *Arch. Pathol.* **53**, 301–314.
Russell, W. O., Ortega, L. R., and Wynne, E. S. 1952. *Cancer Research* **12**, 216–218.
Sabin, F. R., Doan, C. A., and Forkner, C. E. 1932. *J. Exptl. Med.* **56**, 267–289.
Salaman, M. H. 1952. *Brit. J. Cancer* **6**, 155–159.
Salaman, M. H., and Gwynn, R. H. 1951. *Brit. J. Cancer* **5**, 252–263.
Sall, R. D., Shear, M. J., Leiter, J., and Perrault, A. 1940. *J. Natl. Cancer Inst.* **1**, 45–55.
Salzberg, D. A., and Griffin, A. C. 1952. *Cancer Research* **12**, 294.
Sasaki, T., and Yoshida, T. 1935. *Virchow's Arch. pathol. Anat. u. Physiol.* **295**, 175–200.
Saxen, E., and Ekwall, P. 1950. *Acta unio intern. contra Cancrum* **7**, 156–159.
Schabad, L. M. 1930. *Z. Krebsforsch.* **30**, 24–59.
Schabad, L. M. 1935. *Z. Krebsforsch.* **42**, 295–305.
Saxton, J. A., Boon, M. C., and Furth, J. 1944. *Cancer Research* **4**, 401–409.
Schaefer, A. E., Copeland, D. H., Salmon, W. D., and Hale, O. M. 1950. *Cancer Research* **10**, 786–792.
Schinz, H. R., and Uehlinger, E. 1942. *Z. Krebsforsch.* **52**, 425–437.
Seelig, M. G., and Cooper, Z. K. 1933. *Am. J. Cancer* **17**, 589–667.
Seligman, A. M., and Shear, M. J. 1939. *Am. J. Cancer* **37**, 364–395.
Setälä, K. 1949a. *Acta Pathol. Microbiol. Scand.* **26**, 223–233.
Setälä, K. 1949b. *Acta Pathol. Microbiol. Scand.* **26**, 280–295.
Setälä, K., and Ekwall, P. 1950. *Nature* **166**, 188–189.
Shapiro, J. R., and Kirschbaum, A. 1951. *Cancer Research* **11**, 644–647.
Shear, M. J. 1936. *Am. J. Cancer* **26**, 322–332.
Shear, M. J. 1937. *Am. J. Cancer* **29**, 269–284.
Shear, M. J. 1938. *Am. J. Cancer* **33**, 499–537.
Shear, M. J., Stewart, H. L., and Seligman, A. M. 1940. *J. Natl. Cancer Inst.* **1**, 291–302.
Shimkin, M. B. 1939. *Public Health Rept. (U.S.)* **54**, 1388–1391.
Shimkin, M. B., and Andervont, H. B. 1940. *Public Health Rept. (U.S.)* **55**, 537–545.
Shimkin, M. B., and Grady, H. G. 1940. *J. Natl. Cancer Inst.* **1**, 119–128.
Shimkin, M. B., and Lorenz, E. 1942. *J. Natl. Cancer Inst.* **2**, 499–510.
Shimkin, M. B., and Wyman, R. S. 1947. *J. Natl. Cancer Inst.* **8**, 49–52.
Shubik, P. 1950a. *Cancer Research* **10**, 13–17.
Shubik, P. 1950b. *Cancer Research* **10**, 713–717.
Simpson, W. L., and Cramer, W. 1943. *Cancer Research* **3**, 515–518.
Simpson, W. L., and Cramer, W. 1945. *Cancer Research* **5**, 5–10.
Simpson, W. L., Carruthers, C., and Cramer, W. 1945. *Cancer Research* **5**, 1–4.
Smith, D. L., Wells, J. A., and d'Amour, F. E. 1942. *Cancer Research* **2**, 40–44.
Smith, W. E. 1947. *J. Exptl. Med.* **85**, 459–478.
Smith, W. E., and Rous, P. 1945. *J. Exptl. Med.* **81**, 621–646.
Sobolewa, N. G. 1926. *Vestnik roentgenol. i. radiol.* **4**, 191; (Abstr. 1928. *Cancer Rev.* **3**, 116).

Spiegelman, S. 1947. *Cancer Research* **7**, 42–43.
Spitz, S., Maguigan, W. H., and Dobriner, K. 1950. *Cancer* **3**, 789–804.
Stamer. S. 1945. *Acta Pathol. Microbiol. Scand.* **22**, 65–72.
Steiner, P. E., and Falk, H. L. 1951. *Cancer Research* **11**, 56–63.
Stern, K., and Willheim, R. 1943. The Biochemistry of Malignant Tumors. Reference Press, Brooklyn, N.Y.
Stewart, H. L., and Lorenz, E. 1942. *J. Natl. Cancer Inst.* **3**, 175–189.
Strait, L. A., Hrenoff, M. K., and DeOme, K. B. 1948. *Cancer Research* **8**, 231–240.
Strombeck, J. P., and Ekman, B. 1949. *Acta Pathol. Microbiol. Scand.* **26**, 480–495.
Strong, L. C. 1944. *Arch. Pathol.* **37**, 131–135.
Strong, L. C. 1949a. *Brit. J. Cancer* **3**, 97–108.
Strong, L. C. 1949b. *Yale J. Biol. Med.* **21**, 293–299.
Strong, L. C., and Figge, F. H. J. 1946. *Cancer Research* **6**, 466–469.
Strong, L. C., and Smith, G. M. 1939. *Yale J. Biol. Med.* **11**, 589–592.
Sugiura, K. 1942. *Cancer Research* **2**, 770–775.
Suntzeff, V., Babcock, R. S., and Loeb, L. 1940. *Am. J. Cancer* **39**, 56–60.
Suntzeff, V., Burns, E. L., Moskop, M., and Loeb, L. 1938. *Am. J. Cancer* **32**, 256–289.
Suntzeff, V., Carruthers, C., and Cowdry, E. V. 1947. *Cancer Research* **7**, 439–443.
Sutton, R. L. 1938. *Arch. Dermatol. and Syphilol.* **37**, 737–777.
Sutton, R. L. 1942. *Arch. Dermatol. and Syphilol.* **46**, 1–39.
Takizawa, N. 1940a. *Gann* **34**, 1–5.
Takizawa, N. 1940b. *Proc. Imp. Acad.* (*Tokyo*) **14**, 309–312; (Abstr. 1940. *Am. J Cancer* **40**, 410).
Tannenbaum, A. 1940. *Am. J. Cancer* **38**, 335–350.
Tannenbaum, A. 1942a. *Cancer Research* **2**, 460–467.
Tannenbaum, A. 1942b. *Cancer Research* **2**, 468–475.
Tannenbaum, A. 1944. *Cancer Research* **4**, 673–677.
Tannenbaum, A. 1945. *Cancer Research* **5**, 616–625.
Tannenbaum, A. 1947. *Ann. N.Y. Acad. Sci.* **49**, 5–18.
Tannenbaum, A., and Silverstone, H. 1949. *Cancer Research* **9**, 162–173.
Tannenbaum, A., and Silverstone, H. 1952. *Cancer Research* **12**, 744–749.
Tannenbaum, A., and Silverstone, H. 1953. *Advances in Cancer Research* **1**, 451–501.
Tatum, E. L. 1947. *Ann. N.Y. Acad. Sci.* **49**, 87–97.
Taussig, J., Cooper, Z. K., and Seelig, M. G. 1937. *Surg. Gynecol. Obstet.* **66**, 989–993.
Teutschlaender, O. 1923. Strahlentherapie **15**, 812–821.
Turner, F. C. 1941. *J. Natl. Cancer Inst.* **2**, 81–83.
Twort, C. C., and Fulton, J. D. 1930. *J. Pathol. Bacteriol.* **33**, 119–143.
Twort, J. M., and Twort, C. C. 1939. *Am. J. Cancer* **35**, 80–85.
Various Authors. 1947a. *Brit. Med. Bull.* **4**, Nos. 5 & 6.
Various Authors. 1947b. *Ann. N.Y. Acad. Sci.* **49**, *Art.* **1**, 1–140.
Various Authors. 1949. Semaine d'Etude sur le Probleme Biologique du Cancer. Pontif. Acad. Scient. Scripta Varia, Vatican City.
Various Authors. 1952. *Ann. N.Y. Acad. Sci.* **54**, *Art.* **6**, 869–1232.
Vernoni, G. 1952. *Scient. Med. Ital.*, **2**, 395–430.
Vigier, P., and Guerin, M. 1952. *Bull. assoc. franç. étude cancer* **39**, 174–183.
Vogt, M. 1948. *Experientia* **4**, 68–69.
Waddington, C. H. 1935. *Nature* **135**, 606–608.
Watson, A. F. 1932. *Cancer Rev.* **7**, 445–462.
Watson, A. F. 1935. *Am. J. Cancer* **25**, 753–762.
Watson, A. F., and Mellanby, E. 1930. *Brit. J. Exptl. Pathol.* **11**, 311–322.

Weil-Malherbe, H., and Dickens, F. 1946. *Cancer Research* **6**, 171–178.
White, F. R., and White, J. 1944. *J. Natl. Cancer Inst.* **4**, 413–415.
White, F. R., White, J., Mider, G. B., Kelly, M. G., and Heston, W. E. 1944a. *J. Natl. Cancer Inst.* **5**, 43–48.
White, J., and Andervont, H. B. 1943. *J. Natl. Cancer Inst.* **3**, 449–451.
White, J., Mider, G. B., and Heston, W. E. 1944b. *J. Natl. Cancer Inst.* **4**, 409–411.
White, J., and White, A. 1939. *J. Biol. Chem.* **131**, 149–161.
White, J., White, F. R., and Mider, G. B. 1947. *Ann. N.Y. Acad. Sci.* **49**, 41–48.
Williams, R. T. 1947. Detoxication Mechanisms. Chapman & Hall Ltd., London.
Willis, R. A. 1948. Pathology of Tumors. Butterworth & Co. Ltd., London. pp. 106–125.
Wilson, R. H., DeEds, F., and Cox, A. J. 1941. *Cancer Research* **1**, 595–608.
Wilson, R. H., DeEds, F., and Cox, A. J. 1947a. *Cancer Research* **7**, 444–449.
Wilson, R. H., DeEds, F., and Cox, A. J. 1947b. *Cancer Research* **7**, 450–452.
Woglom, W. H. 1926. *Arch. Pathol.* **2**, 533–576 and 709–752.
Wolbach, S. B. 1936. *Arch. Pathol.* **22**, 279.
Wolbach, S. B. 1937. *Am. J. Pathol.* **13**, 662–663.
Wolf, G. 1952. Chemical Induction of Cancer. Cassell & Co. Ltd., London.
Woolley, G. W., and Little, C. C. 1945a. *Cancer Research* **5**, 193, 203–210.
Woolley, G. W., and Little, C. C. 1945b. *Cancer Research* **5**, 211–219.
Yamagiwa, R., and Itchikawa, K. 1914. *Verhandl. d. jap. pathol. Ges.* (cited by Woglom).
Young, L. 1950. *Biochem. Soc. Symposia* (*Cambridge, Engl.*) No. **5**, 27–39.
Zondek, B. 1941. Clinical and Experimenta Investigations of the Genital Functions and Their Hormonal Regulation, pp. 1541–159. Williams & Wilkins Co., Baltimore.

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Ionizing Radiations and Cancer

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CONTENTS

I. Introduction

Ionizing radiation has several important relationships to cancer. Two of these, the production of cancer by radiation and the destructive effects of radiation on the neoplastic process, will be discussed in some detail in this review. The effect of radiation on tumor immunity will also be mentioned. The use of tracers in delineating the details of tumor metabolism will be discussed only in so far as radiation effects are concerned, although the fact that tracer methods make it possible to study synthetic processes in a direct way indicates that this branch of cancer research has a bright future.

The history of this subject, like that of cancer research in general, is virtually limited to the present century. The limitation is even more strict in that it dates of necessity from the discovery of x rays and radium. Since their practical usefulness and the severity of their effects on tissue were soon recognized, we find that early attention was given to effects on tissue and to carcinogenesis. Unfortunately, during the first half of this period measurements of radiation dose lacked the present degree of precision, so that data from this period are mainly semiquantitative. The early history of observations in radiation carcinogenesis was outlined in a previous review (Brues, 1951a).

II. Basis of Radiation Effects

It is likely that much further understanding of radiation effects on cells and tissues will have to be gained before the processes involved in the production or therapy of cancer are unraveled. It seems worth while to restate here certain facts in relation to radiation effects that may have important bearing on cancer research.

1. Immediate radiation effects are largely chemical in nature and are localized in space. Probably the most important of these from the point of view of pathology is the production of short-lived free radicals in water, whose major effects are oxidative. Disruption of other molecules through excitation or transposition of electrons also occurs, but, as far as we know at present, this is of secondary importance in radiotoxic action on the higher organisms.

Ultraviolet irradiation, which will not be considered at any length here, requires the absorption of much more energy in, for example, carcinogenesis in skin. Because of the low penetration of ultraviolet light through tissue, its effects in the higher organisms are strictly limited.

The effects of radiation are, by and large, entirely attributable to the irradiated areas. Where remote effects occur they may be treated as exceptions, and it is profitable in these cases to give special consideration to the chain of events connecting the local insult to the later response. The localized nature of the effect is particularly demonstrated in radiation carcinogenesis; the chief exceptions to this will be discussed below.

2. The various biological effects of ionizing radiations take place over a remarkably wide range of dosages. Denaturation of proteins and killing of certain of the simplest organisms require a dose of the order of 1,000,000 r (roentgen units). Immediate death of the higher animals "under the beam" does not occur until 50,000 r or more have been absorbed. Dosages of x radiation between 200 and 1000 r are lethal to the higher animals (depending on the species) after a latent period of several days to three or four weeks, however, whereas leucopenia and visible damage to chromosomes are detectable after less than 100 r.

Radiation cancer usually occurs after dosages of 1500 to 5000 r or more, and the dosages useful in cancer therapy are of the same order. A great deal of attention has been given by clinical radiologists to the relative radioresistances of the several types of malignant tumors. Generally speaking, the radiosensitivities of tumors fall in the same range as those of the normal tissues.

3. Clinical radiation sickness after total-body irradiation can largely be explained on the basis of cellular damage to the more radiosensitive tissues. This may not be true of the initial radiation response, which, al-

though familiar to radiotherapists, is less understood in its fundamentals than the later responses. The gradual development of a severe reaction state several days after irradiation (true radiation sickness), and the course of recovery therefrom in nonfatal cases, is associated with depressed function of the blood-forming organs and damage to the epithelial lining of the digestive tract, while such benign changes as epilation and sterility are likewise based on obvious cellular damage. Present indications are that therapeutic procedures favoring recovery from radiation sickness are largely supportive in nature and act through restoration of blood volume, improved recovery of leucocytes, or control of bacteremia. Certain prophylactic measures, on the other hand, may be directed towards influencing the state of tissue oxidations at the time of irradiation.

4. Damage on the cellular level seems basic to most other radiation effects: cytoplasm and nucleus both partake in the effects, but the nuclear components appear to be most reproducible and occur at the lowest dosages. The visible nuclear consequences are an immediate inhibition of cell division and damage to chromosomes resulting in subsequent abnormal divisions. Owing to the fact that the more radiosensitive tissues—in general those that are in a process of continual restoration—show the most marked nuclear changes, it is generally assumed that those changes are in large part responsible for such destruction.

A considerable number of chemical radiomimetic agents have recently been discovered which have predominantly nuclear effects. These substances are also, in various degrees, carcinogenic, carcinolytic, and mutagenic. The alkylamines (nitrogen mustards), in particular, also have toxic actions quite similar to those of total-body irradiation. Certain antimetabolites, including those interfering with folic acid synthesis, inhibit tissue proliferation and the growth of the more radiosensitive tumors. It is an unfortunate fact that carcinolytic and tumor-inhibiting agents in these general categories are generally effective only against such tumors as are particularly sensitive to the ionizing radiations.

III. Radiation Carcinogenesis

The production of cancer by local irradiation antedates perhaps the discovery of ionizing radiation as such. The *Bergkrankheit* of the workers in the mines at Schneeberg and Joachimsthal has been thought to be related to the small concentration of radon in the air of these mines (Hueper, 1942); and, although direct experimental evidence is lacking for the carcinogenic role of radiations in this instance, it is nevertheless true that the air passages of miners breathing this air are subjected to α radiations well above presently accepted "permissible" levels for the gas and its decay products (Evans and Goodman, 1940).

More definite clinical evidence for radiation carcinogenesis lies in the production of skin cancer or subcutaneous sarcoma in heavily x-irradiated areas (Hesse, 1911); of malignant bone tumors after prolonged retention of radium or radium plus mesothorium (Martland and Humphries, 1929) or after local x irradiation (Cahan *et al.*, 1948); of pelvic tumors after irradiation in this area (Fournier, 1935); and of laryngeal tumors after roentgen treatment of Graves's disease (Petrov and Krotkin, 1932). Regular exposure to moderate or desultory exposure to excessive total-body radiation is thought to be responsible for the high incidence of leukemia in physicians and particularly among radiologists (March, 1947), and that this may follow even a single radiation dose is suggested by the modest increase in the incidence of leukemia among the survivors at Hiroshima who were in the areas closest to the atomic bomb.

Local irradiation in various sites has been widely investigated, and the production of neoplasia has been abundantly verified. Many of these studies have been reviewed previously (Brues, 1951a; Salter, 1948) and will not be covered in detail here. Earlier work dealt with the effects of locally directed x radiation and the implantation of radon tubes and other radiation sources. More recently, the *skin and subcutaneous tissues* of rats and mice were exposed uniformly to β radiation from P^{32} (Raper *et al.*, 1951). This was done by placing the animals in boxes made of phosphorus-containing Bakelite plaques that were activated by pile neutrons. This resulted in the development of skin carcinomas and a small proportion of subcutaneous sarcomas, corresponding roughly to the fractions of β-ray energy absorbed, respectively, in the epithelium and in the subcutaneous tissues. The optimal dosages for induction of malignant tumors was 4000 to 5000 rep (equivalent roentgens), and tumors began to appear only after a latent period of about nine months. Under these conditions, where the entire body surface was irradiated, multiple tumors were observed (up to forty or more in a rat), so that a single animal would bear tumors of a variety of histologic types. The employment of daily β-ray treatments of 50 rep produced very similar results. Unfortunately, it is impossible to make a quantitative comparison between single and daily dosages, since no daily dosage levels were used between 50 rep and 5 rep (the latter accumulating to about 1800 rep in a year without inducing tumors in this period).

Subcutaneous tumors have been produced by a variety of other techniques causing local concentration of ionizing radiation. Local implantation of radon tubes and other radioactive sources have produced various tumors; Hartwell (1951) has tabulated many of the earlier data. In a series of experiments in this laboratory, plutonium and insoluble salts of Y^{91} were injected subcutaneously and intramuscularly in mice. A very

high percentage of the mice receiving injections of 1 to 30 μc. of Y^{91} phosphate or 0.06 to 1 μc. of Pu^{239} developed local fibrosarcomas (Lisco *et al.*, 1947b). These tumors appeared after a long latent period (about 200 days) and increasing the dose through an additional factor of 10 had no influence on the length of this intervening period.

Gastrointestinal tumors have been produced by isolated exposures of this tract in a manner analogous to administration of β rays externally to the skin. When solutions of Y^{91} are fed to rats or mice by stomach tube, virtually none of the isotope is absorbed; and the β-ray dose to the colon is greater than to the rest of the tract because of the fact that the gastrointestinal contents move more slowly through the lower part of the tract. In conformity with this distribution of local dosage, adenocarcinomas were induced in the colon by single sublethal feedings or by daily feedings over a period of months (Lisco *et al.*, 1947a). Again, the latent period was one of many months. The earliest tumor was seen 135 days after a single feeding, and the mean latent period was over a year. Although many of these animals gained weight normally, ulcerative and hyperplastic changes were generally observed even in animals that did not develop tumors.

Local irradiation of the skeleton is accomplished through fixation of a large number of radioelements. Elements in the alkaline earth group (notably Ca^{45}, Sr^{89} or Sr^{90}, and Ra) are deposited in bone with extreme efficiency, yet are lost very slowly after the blood concentration is reduced. Many other elements, notably gallium, the rare earths, and the heaviest elements, also appear in the skeleton in higher concentrations than elsewhere in the body.

The most nearly quantitative data on human carcinogenesis are derived from observations on the occurrence of bone tumors after absorption of radium or mixtures of radium and mesothorium. Bone sarcoma has frequently been seen several years after fixation of radium, which was administered for therapeutic purposes or ingested in the course of painting luminous watch dials. Because very small amounts of radium deposited in the human skeleton can be detected by physical methods, estimates of the retained radium have been readily obtained in such cases. Clinical surveys have indicated that sarcoma may occur when as little as 1 μg. of radium is present in the skeleton.

What this means in terms of total radiation during the retention period is less certain, since few patients have been studied over long periods of time. After ten to twenty years, the daily excretion rate may be as little as 10^{-5} of the retained amount, and the amount present at twenty years is about one-fifth that at six months (Marinelli *et al.*, 1952). Moreover, many patients (including those in the watch-dial industry) also received meso-

thorium$_1$ (an isotope of radium), which has a decay chain of α-ray emitters somewhat better retained than that of radium but reduced about tenfold after two decades by physical decay. It has been pointed out that most of the cases bearing sarcomas with a radium burden under 5 μg. had also been exposed to considerable amounts of this shorter-lived chain (Aub *et al.*, 1952). A recent study has indicated that patients receiving pure radium and retaining as little as 1 μg. after twenty years have roentgenologic evidence of skeletal damage (Marinelli *et al.*, 1952). In any case, it may be estimated that the accumulated dose of radiation to the skeleton in cases showing pathologic changes and tumors has always been in the thousands of rep.

A series of cases of bone sarcoma apparently induced by x-ray therapy has been collected (Cahan *et al.*, 1948), showing that 1500 r (based on external measurements) may be enough to evoke a carcinogenic response several years later. Owing to the relatively high mean atomic number of osseous tissue, the physical dose to bone is somewhat higher than to soft tissues.

Experimental studies of animals have shown that bone tumors are readily induced by a number of bone-seeking radioelements, including radium (Sabin *et al.*, 1932; Dunlap *et al.*, 1944; Brues *et al.*, 1946), strontium 89 (Brues *et al.*, 1946), cerium 144–praseodymium 144 (Lisco *et al.*, 1947b), plutonium (Lisco *et al.*, 1947b), and phosphorus 32 (Brues *et al.*, 1949; Koletsky *et al.*, 1950). Undoubtedly any radioelement concentrating primarily in the skeleton would be carcinogenic if administered in adequate dosage. Latent periods are long (under optimal conditions, five to eight months), as in other instances of radiation carcinogenesis.

Lung tumors follow the introduction of radioelements by tracheal intubation (Lisco and Finkel, 1949). They are found in association with severe local radiation damage to the lung.

IV. Mechanism of Carcinogenesis

Leaving for the moment the question of the neoplastic consequences of total-body irradiation, which will be discussed in a later section, it appears that the induction of tumors by local irradiation is remarkably reproducible and that its study should lead to important information concerning the mechanism of carcinogenesis in general. In so far as studies have been made, there seems to be no great diversity in the response of various species of mammal to such stimuli, as regards dose-effect relationships or the nature of the response. In chemical carcinogenesis, of course, it is known that there are differences in the metabolic fate of various carcinogens that depend on inborn and environmental factors, which may to some extent determine differences in carcinogenicity.

It seems established that continued stimulation is not necessary to evoke a neoplastic process in the case of ionizing radiations. With ultraviolet irradiation of skin, on the other hand, Blum (1950) has shown in the course of exhaustive experimental work that repeated stimulation is necessary. In the case of chemical carcinogens, it is often true that the greater part of the effective material is rapidly eliminated after administration, yet recent work (Miller, 1950; Miller and Miller, 1947) suggests that a small amount may persist in combination with tissue proteins, and this may be necessary for carcinogenesis. The most clear-cut instance of a response after a brief period of intense stimulation is in the case of skin cancer after a single superficial β-ray treatment by several months. The somewhat scanty data that are available indicate that daily treatments are no more efficient and may be somewhat less efficient as regards total radiation dose required for a given response.

The length of the latent period in radiation carcinogenesis is undoubtedly of significance. Although this period is comparable with that seen in most instances of chemical carcinogenesis, it has never been possible—even under the most intense stimulation—to shorten it to the few weeks characteristic of maximal stimulation of a susceptible animal by a suitable chemical carcinogen, in which the latent period can virtually be accounted for by the growth of a few cells to a tumor of visible size.

Since some intermediate factors must exist between application of a radiation stimulus and the onset of tumor growth (at least at a growth rate equal to that of the established tumor), efforts have been made to show that the process is mediated by chemical events taking place in tissue as a result of irradiation. A comparison of pure cholesterol with the same material after intense pile irradiation (at least equivalent to 10^8 r) has shown that the irradiated cholesterol, although altered markedly in its chemical structure, had not gained in carcinogenicity (Cloudman *et al.*, 1952).

Similar experiments were performed at an opposite extreme of carcinogenicity, in which potent hydrocarbon carcinogens were similarly irradiated, with a possible increase in their biologic activity (Barnes *et al.*, 1948). An earlier report indicated a synergistic action between methylcholanthrene, and cosmic radiation (Figge, 1947), but this has so far failed to be generally confirmed (George *et al.*, 1949). Likewise, a direct combination of β irradiation and carcinogenic hydrocarbons to stimulate tumor genesis in skin and subcutaneous tissues has failed to elicit more than a simple additive response (Cloudman and Hamilton, 1949).

A comparison between the histogenesis of skin tumors induced by β irradiation (7900 rep) and by benzopyrene (Glücksmann, 1951) has served to emphasize the difference in the latent periods. Whereas after benzopyrene the tumors seem to arise directly in stimulated epithelial cells, β

irradiation results in a cyclical hyperplasia and necrosis (attributed in part to vascular damage), and tumors arise after several months, when the hyperplastic responses have become less vigorous. It is noteworthy that these observations were made after a single irradiation of thirty seconds' duration. In like manner, the origin of tumor foci in bone irradiated by radium or plutonium appears to follow a series of destructive and proliferative changes (Bloom and Bloom, 1949).

Thus, both statistical and histologic investigations indicate that the process of radiation carcinogenesis is a rather complicated one and probably progresses in more than one stage. On the other hand, there is much to suggest that a somatic mutation hypothesis will satisfy most of the known facts regarding the nature and origin of cancer, so that the status of this theory is worthy of some discussion.

V. The Mutation Hypothesis

A malignant tumor, once it has developed, consists of a strain of self-propagating cells that have developed certain deviant characteristics which must undoubtedly be reducible to a chemical or metabolic basis. The temptation is therefore strong to assume that the origin of cancer is in some way related to a discrete chemical change, possibly occurring in a single somatic cell (similar to mutation in a germ cell), which confers such a property on this cell and its descendents.

In favor of this view are the facts that many of the known carcinogens have also been shown to act as mutagens and that many of them also have visible effects on the nucleus and on the course of cell division. Certainly the frequency with which chromosome aberrations and other defects in cell division are seen in somatic cells after relatively small radiation dosages indicates that deficiencies and maldistribution of genic material is a necessary consequence in most cells, at least in a proliferating tissue, irradiated with dosages in the order of thousands of roentgens.

If we assume, for the moment, that it is sufficient for a single cell to undergo a particular genic injury in order to mutate to a cancer cell and that such a cell will inevitably lead to a tumor through proliferation, some interesting consequences follow. It can be shown that under optimal conditions for radiation carcinogenesis not more than one in 10^7 or 10^8 cells treated in equivalent fashion (by thousands of rep of β radiation) gives rise to a tumor. It seems clear that tumors arise focally, and this is borne out by the fact that under an intense stimulation, such as the administration of sublethal doses of Sr^{89}, the distribution of multiple tumors in a group of mice follows the random Poisson distribution (Brues, 1949). The probability of carcinogenesis on the cellular level is thus of the same order as that of a single genetic mutation induced by irradiation. When we

progress from the mouse to the rat and the rabbit, in which equivalent dosage:body weight remains the same, many more cells will receive the same degree of stimulation without a comparable increase in number of tumors; and in point of fact there is some evidence that the latent period is increased in species of longer life span (Brues, 1951b). Recalling the fact that the rabbit has, tissue by tissue, some one-hundred times as many cells as the mouse, we see at once that, if single cell mutations were responsible for tumor formation, cells of the larger animals must be correspondingly unlikely to mutate in this manner, in spite of their morphological similarity. This *a priori* improbable situation might, in a sense, be a necessary adaptation in the evolution of larger animals, but one recalls that there is evidence to suggest that genetic mutation frequency is not reduced in man, but may actually be increased (Neel and Falls, 1951).

The whole question of the possible role of somatic mutations is consequently one to which answers are lacking at present. The weight of the evidence seems to indicate that other factors, at least general tissue responses, probably including vascular changes, are a necessary part of the radiation carcinogenic process as it is usually seen.

VI. Some Practical Matters

Since tumor induction shares with genetic effects the distinction of occurring at the lowest radiation dosages known to alter normal conditions, one question of practical importance is whether (as is probably the case with mutations in the germ plasm) no true threshold exists; that is, whether even the smallest dosage of radiation confers a probability of tumor formation proportional to its amount. If this is the case, then there is no point in setting a "permissible" dose in terms of one which will have no effects. It is possible that this is the situation, although there are no clinical or experimental data that clearly indicate that a carcinogenic process takes place in the absence of some grossly visible overall pathologic process. Further work should be directed toward the question of whether there is a threshold, especially since the future may bring mass irradiations of populations, where a statistical tendency not seen in past clinical or experimental work might emerge.

Another question which has been raised relates to the problem of radioactive dust particles. Since they form an industrial hazard, it is desirable to know whether concentrated "hot spots" of radiation are more effective carcinogenically than the same amount of radiation distributed diffusely. A recent experiment (Passonneau *et al.*, 1953) employed rats exposed to equivalent amounts of β-ray energy infringing on the body surface either diffusely from a plane source or from ten to fifty point sources distributed over the same area. The results indicate that the point

sources are actually less effective in inducing skin cancer than the plane source emitting 5000 or 7500 rep. This can perhaps be explained by the fact that a larger proportion of irradiated cells are killed where the fewer, more intense sources are used. This experiment therefore fails to settle the fundamental question, namely, whether or not the probability of tumor development in a given tissue of a given species or strain is linear with dose.

VII. Carcinogenic Actions of Total-Body Irradiation

Unlike most active agents, the ionizing radiations administered in the form of external or γ radiation or neutrons of high energy may subject the tissues to a relatively uniform physical and chemical dosage. Many investigators have demonstrated carcinogenic action by single sublethal dosages or by dosage patterns that permit animals to live long enough to pass the latent period. Scrutiny of the data makes it appear that in some instances a general but mild carcinogenic stimulus is involved, whereas in others there are obviously intermediate physiologic factors.

The general carcinogenic effect of total-body irradiation is apparently correlated with a reduction in life span (Lorenz, 1950), whether a single sublethal dose is given early in life or a low dosage rate is continued throughout the experiment. Isolation of the various "causes" or pathologic states associated with death indicates that many of these appear earlier in irradiated than in control animals (Sacher *et al.*, 1949), although this should not necessarily be taken to indicate that the radiations act through acceleration of the aging process. The result is, of course, statistically the same. If we represent the "rate of aging" as the reciprocal of the life span, we find that this quantity is increased about linearly with the dose of radiation (Boche, 1946) within the range of dosage that can yield significant results (suggesting, but not proving empirically, the absence of a threshold).

Increased general tumor incidence has been shown to follow neutron irradiation as well as x irradiation of rats (Barnett, 1949; McDonald *et al.*, 1947). Induction or acceleration in the appearance of lung tumors in mice also follows total-body irradiation and appears attributable to local effects (Lorenz, 1950).

We now turn to two instances of carcinogenesis in mice where the evidence definitely suggests intermediate physiologic factors; that is, where a direct relation between irradiation of a single cell or locus and development of cancer can be ruled out. The first of these is the case of *ovarian tumors* in mice. Furth and Butterworth (1936) first observed that these tumors followed, after several months, a single sublethal total-body irradiation. Subsequent observations (Lick *et al.*, 1949) have indicated that

there is no such effect if a single ovary is irradiated, provided that there is present at the same time an intact ovary that was shielded during the irradiation. This strongly suggests that the primary effect of irradiation on the ovary may be to cause it to evoke a gonadotropic response which results in tumor development; the analogy to the behavior of ovarian transplants into the bed of the portal circulation (Furth and Sobel, 1947) is obvious. Lorenz finds that mice receiving 0.11 r daily develop considerable numbers of ovarian tumors and that those with granulosa-cell tumors are also highly prone to develop mammary sarcoma, a neoplasm that is ordinarily very rare in the strain of mice used (Lorenz, 1950).

Mouse leukemia or lymphoma is another neoplasm that follows relatively small dosages of total body irradiation. This is a spontaneous disease that occurs frequently in many strains of mice, and its morbidity rate increases with age in the same manner as that of most spontaneous tumors. The evidence for the leukemogenic action of irradiation in mice is considerable and has been reviewed previously (Brues, 1951a). Some recent observations make it clear that unknown physiologic factors must be important in this instance. Age at the time of irradiation is very critical in some strains (Kaplan, 1948b); tumors in young mice tend to arise in the thymus (Kaplan, 1948a) and are partly prevented by thymectomy (Furth, 1946); and total-body irradiation is enormously more effective than the summation of responses to partial-body irradiations would indicate (Kaplan, 1949). Moreover, when two half-body irradiations are given separately, the time interval between these irradiations (within a span of days) strongly determines their additivity (Kaplan, 1951). The protection that is afforded by protecting part of the lymphoid tissue indicates that other factors must modify to a considerable extent any local cellular changes induced by irradiation. Furthermore, it has been shown that fractionation of the total-body dose into ten daily dosages results in an increased response, perhaps the only case in which enhancement of a carcinogenic effect of ionizing radiation through fractionation has been shown. Phosphorus 32, which irradiates all the blood-forming tissues, is leukemogenic to mice (Furth and Butterworth, 1936) whereas radiostrontium, which spares most of the lymphoid tissues, is not (Brues *et al.*, 1946).

Another fact indicating that genetic or physiologic factors are of importance in determining radiation leukemia in mice lies in lack of correlation, among various strains, with the intensity of the leukemogenic response to other agents and with the normal incidence in these strains (Kirschbaum and Mixer, 1947).

It is worthy of comment that the induction of mouse leukemia takes place through a shorter latent period than the other known carcinogenic responses.

Other species of animals appear to be much less prone to develop radiation leukemia. Evidence cited above suggests that the human being is mildly susceptible and that the latent period is several years.

VIII. Factors in Radiation Therapy of Tumors

Despite the fact that the major usefulness of ionizing radiations, excepting radiography and some recent developments, has been in the therapy of human tumors, we are still far from understanding their mode of action in causing tumor regression. Regression of irradiated tumors can involve a number of processes; damage to resting cells, death of cells at a subsequent mitosis, cytologic changes because of somatic mutations or unequal distribution of chromatin, inhibition of mitosis, cell differentiation, vascular damage resulting from irradiation of the tumor or of surrounding tissues, and the nature of the repair processes. It is not improbable that all of these processes have some part to play.

Damage to resting cells, or at least visible damage, is probably a minor factor in tumor therapy, where dosages are seldom in excess of 10,000 r. When much higher dosages are used, cell death appears to follow marked volume changes (Buchsbaum and Zirkle, 1949) or other morphologic changes (Brues and Stroud, 1951), while pycnosis of the resting nucleus may occur at about 250,000 r (Tahmisian, 1949).

Death of cells that have been previously irradiated with dosages in the therapeutic range is likely to occur at the time when division takes place. A striking example of this is the simultaneous pycnosis that occurs at the time of beginning cell activity in grasshopper embryos at the end of diapause, although they were irradiated long before (Tahmisian and Adamson, 1951). At times one can observe death of cells after an unsuccessful attempt at division where a chromosome bridge is present (Brues and Rietz, 1951a); this represents a small proportion of the degenerating cells in most injured tissues. The possible role of effects on the genes in determining this has been discussed by Lea (1947, pp. 341ff). Where cell degeneration is a prominent feature of tumor regression, one finds that it takes place only gradually during a period of several hours to days after irradiation (Glücksmann, 1946; Glücksmann and Spear, 1949).

Using chromosome bridges or breaks as a criterion of potentially lethal cell damage, it has been shown that many biological objects are most sensitive in the early stages of mitosis (Sparrow, 1948). It is of interest that mouse lymphoma, which is a tissue of high radiosensitivity, appears sensitive, in terms of chromosome damage, over a longer period of its mitotic cycle than most other materials (Marshak, 1942).

Inhibition of mitotic division is a universal accompaniment of even moderate radiation dosages, and Lea (1947, p. 300) suggested that it

might account for the considerable enlargement of cells following irradiation. It is possible that cell enlargement plays a more important role in the regression of tumors than has been suspected, since it seems to be most noticeable at the time when regression is actively taking place (Brues and Rietz, 1951a), and hence at a time when pathologic examination of human material is not often made. It does occur in human neoplasms (Wood, 1949) and has also been observed in experimental tumors several days after a single radiation dose (Tansley and Wilson, 1947) or after intermittent treatments. Using an imbedded point source of β irradiation, we have noted that enlargement of tumor cells occurs in just those areas where the dosage has been high enough to result in a continuous inhibition of cell division, which offers strong evidence that it results from continued nuclear and cell growth in the absence of mitosis (Brues and Rietz, 1951b).

It is to be remembered that some nuclear or cell swelling may take place soon after irradiation, possibly because of physiological mechanisms similar to those causing cell death where extremely large doses are given. Failla (1940) observed vacuolation of cells soon after irradiation, and a detailed account of the phenomena has been made by Warren *et al.* (1951). Cytochemical evidence has shown that this process may be associated with depolymerization of desoxyribonucleic acid (Harrington and Koza, 1951), which has also been observed by gross chemical methods several hours after irradiation of the thymus (Ely and Ross, 1949). It appears that further work will be necessary to permit a clear separation of the early changes in irradiated cells from the more gradual enlargement resulting from inhibited mitosis [with synthesis or endomitosis (Tansley and Wilson, 1947) going on]. It is likewise not possible at present to explain the relation of the latter phenomena to tumor regression.

Cell differentiation is another response which has been brought into consideration as a possible radiation effect leading to the cessation of tumor growth. As described by Glücksmann (1946), the differentiating cell is one with a large amount of differentiating cytoplasm and a relatively small nucleus, permanently incapable of division. The development of cells of this type during irradiation, as well as their presence in the untreated tumor, is well correlated with curability (as distinguished from regression rate) after radiation therapy (Glücksmann, 1948). Cells appear during therapy in tumors of a given cell type which take on certain characteristics of the parent cells; thus, enlarging cells of an epithelial tumor keratinize, and persisting bone tumor cells begin to lay down calcium in a pattern resembling the structure of bone (Glücksmann, 1952). Responses of this type have been noted frequently in human tumors and normal tissues under radiation therapy; a recent report describes maturation and keratinization in carcinoma cells (Hall and Friedman, 1948), and "over-

differentiation" in the form of excessive collagen formation in connective tissue is also noted (Jolles, 1949). Other tissue responses, such as squamous metaplasma in the normal oral mucous glands (Friedmann and Hall, 1950), may likewise be related to a similar mechanism.

Although the usefulness of differentiation as a criterion of radiocurability may be in dispute, and although the validity of the term "differentiation" in this connection is seriously questioned (Jolles and Koller, 1950), the various changes included here deserve careful and controlled examination. Since acute inhibition of mitosis in tissues of many kinds is universally a temporary phenomenon, and apparently occurs without other cell changes, one must look elsewhere for a mechanism to explain the development of permanently inhibited cells. The exact nature of the "differentiation" of malignant or normal cells under irradiation deserves study not only in connection with the problem of tumor therapy, but for its probable bearing on important and obscure questions in basic cell biology.

Extrinsic Factors. It is recognized that tumor regression may be related to events other than those directly affecting the tumor cells. A recent review (Marinelli and Brues, 1953) discusses these responses in somewhat more detail than will be given here.

Pathologic changes in the *blood vessels* are often encountered in heavily irradiated tissues, including dilatation, thrombosis, and necrosis (Hall and Friedman, 1948). Vascular changes are especially noteworthy in the area surrounding a point source of β irradiation of about 1 mc. (Brues and Rietz, 1951c) and apparently account for the central area of necrosis, since the cells immediately surrounding such an area appear healthy. There have been contradictory assertions: (1) that these changes are fully responsible for radiation effects on tumors (Pullinger, 1932); and (2) that they are in no way responsible (Melnick and Bachem, 1937).

The *connective tissue response* has long been considered important in tumor radiotherapy (Melnick and Bachem, 1937). It is generally believed that the growth of tumors is dependent on the tissue environment, and particularly on surrounding connective tissue, although direct evidence on this point has been difficult to obtain. Certainly there is often considerable difference in the viability and growth rates of metastases from malignant tumors; and the question of the environment of early tumors is bound to enter into any discussion of carcinogenesis. A recent investigation of the relation of diet to tumor responses to radiation (Elson and Lamerton, 1949; Devik *et al.*, 1950) has shown that, although a low protein diet favors immediate inhibition of tumor growth by irradiation, a high protein diet is more favorable for permanent elimination of the tumor; it is suggested that the latter process is supported by a more vigorous con-

nective tissue response. This response has been studied in some detail by the "sieve" technique of irradiation (Jolles and Koller, 1950; Jolles, 1949), which also offers some promise of usefulness in therapy through partial protection of the stroma. The general status of the connective tissue responses, which have a long history, is outlined by Jolles and Koller (1950).

In vitro irradiation has been used in attempts to evaluate the various factors in the irradiation response of tumors. Careful comparative studies of carcinoma *in vivo* and in tissue cultures (Lasnitski, 1945, 1947) suggest that the lethal process is quite comparable except that, at higher dosages, effects appear in the *in vivo* irradiated tumor that are not paralleled in culture and suggest some influence of extracellular factors. Investigation of the death of cultures after a single irradiation emphasizes that the length of time during which a culture remains viable varies with dose and may become very long after low dosages but that death takes place within a few weeks (Paterson, 1942). In the writer's experience, the survival time of irradiated cultures is sufficiently variable to make this criterion of radiation dosage a difficult one to use.

Irradiation of tumor fragments before inoculation again yields somewhat variable results, and it often appears that fragments are somewhat more resistant to irradiation than tumors *in vivo*. Crabtree and Cramer (1932) noted that the radiosensitivity of tumor fragments was increased by treatment with cold or cyanide during the time of irradiation.

It has recently been suggested (Hall *et al.*, 1952) that some of the vagaries of irradiated tumor fragments can be explained on the basis of oxygen tension. It is known that radiosensitivity can be decreased by anoxia in such different systems as bacteria, pollen, and the higher animals, an effect which is no doubt related to the chemical events occurring in water during irradiation. It now appears that the radiosensitivity of tumor fragments is likewise enhanced by conditions that facilitate penetration of oxygen (as in the irradiation of small fragments) or depress oxygen utilization (as do cyanide and low temperatures) and that, under comparable conditions favoring radiosensitivity, they become resistant if irradiated in an oxygen-free environment.

The bearing of these facts on radiosensitivity of tumors *in vivo* is largely unexplored, although it is well known that such variables as temperature and blood supply have marked effects on responses to external irradiation.

An important clinical aspect of radiation therapy is the increasing radioresistance of tumors during the course of repeated irradiation therapy. The gradual development of vascular insufficiency after x-ray therapy offers at least a partial explanation of this phenomenon. Acquired

radioresistance has been discussed in its various aspects by Windholz (1947).

IX. Immunity to Heterologous Tumors

Another matter that is worthy of further investigation is the effect of irradiation on tumor immunity. It seems to be generally true that those tumors that grow appreciably when transplanted into heterologous hosts are the ones that maintain a rapid enough growth rate to outstrip the development of the immune mechanism. The effect of total-body irradiation is to accentuate the tolerance of a host for a heterologous tumor transplant. Since the lymphocyte seems to play an important role in tumor immunity (Murphy and Sturm, 1925; Ellis *et al.*, 1950), it may be presumed, as a working hypothesis, that irradiation acts through depression of the lymphocyte reserve or of immune material derived from these cells, probably the former (Kidd, 1950). The administration of 100 r total-body irradiation has a considerable effect on heterotransplantability (Hall, 1952), indicating that a radiosensitive system is involved. Local irradiation of an implantation site may impede transplantability; this is apparently not an immune response, since metastases are not so inhibited (Grynkraut and Flaks, 1938). The observation made some years ago (Bagg, 1938) that the resistance of a host may change oppositely, depending on the dosage of radiation, may offer an explanation of paradoxical results in this area of investigation.

X. Isotopic Tracer Studies in Cancer

It is not the purpose of this review to discuss the tracer approach to cancer biochemistry, except to note that this method seems certain to yield critically important information not derivable by other techniques. This is because tracer methods afford the best direct approach to investigation of synthesis of substances in tissue; and it seems most probable that the biochemical peculiarities of cancer involve derangements of some of these synthetic mechanisms.

A word may be in order, however, regarding the validity of experiments employing radioisotopes, from the point of view of the radiation dosage encountered in the tissues under investigation.

A useful formulation of radiation dosage from an isotope distributed uniformly throughout an infinitely large mass of water or tissue (for β-ray emitters, this is a matter of a few grams) is the following:

$$D = 55CE$$

where D is the dosage rate in roentgen equivalents per day, C is the concentration of the isotope in microcuries per gram, and E is the energy of

its radiation in million electron volts. For the C^{14} β ray, this means that a concentration of 1 μc. per gram will yield about 3 rep per day in an infinite volume (less in a limited one), and P^{32} will yield a little over ten times as much.

When we examine the sensitivities of biochemical systems to irradiation, we find that it requires of the order of 300 r or less to reduce markedly the rate of synthesis of desoxyribonucleic acid in tumors and growing tissues (Hevesy, 1951), while the sensitivities of the sulfhydryl enzymes in the pure state are of the same order; and those of many other enzymes are much less (Barron and Dickman, 1949). In case of doubt as to the validity of experimental results, it may be suggested that an experiment be run at two isotope concentrations differing, say, by a factor of 10; if the results are the same, there exists a strong presumption that they are valid for a nonirradiated system.

REFERENCES

Aub, J. C., Evans, R. D., Hempelmann, L., and Martland, H. S. 1952. *Medicine* **31**, 221.

Bagg, H. J. 1938. *Am. J. Roentgenol.* **40**, 418.

Barnes, B. F., Freytag, F. C., Garrison, W. M., and Rosenfeld, I. 1948. *Science* **108**, 82.

Barnett, T. B. 1949. U.S. Atomic Energy Commission Document AECD-2614.*

Barron, E. S. G., and Dickman, S. 1949. *J. Gen. Physiol.* **32**, 595.

Bloom, M. A., and Bloom, W. 1949. *Arch. Pathol.* **47**, 494.

Blum, H. F. 1950. *J. Natl. Cancer Inst.* **11**, 463.

Boche, R. D. 1946. U.S. Atomic Energy Commission Document MDDC-204.*

Brues, A. M. 1949. *J. Clin. Invest.* **28**, 1286.

Brues, A. M. 1951a. *Advances in Med. Phys.* **2**, 171.

Brues, A. M. 1951b. Argonne National Laboratory ANL-4625, p. 106.

Brues, A. M., Lisco, H., and Finkel, M. P. 1946. U.S. Atomic Energy Commission Document, MDDC-145.*

Brues, A. M., and Rietz, L. 1951a. *Ann. N.Y. Acad. Sci.* **51**, 1497.

Brues, A. M., and Rietz, L. 1951b. *Cancer Research* **11**, 240.

Brues, A. M., and Rietz, L. 1951c. Unpublished observations.

Brues, A. M., Sacher, G. A., Finkel, M. P., and Lisco, H. 1949. *Cancer Research* **9**, 545.

Brues, A. M., and Stroud, A. N. 1951. *Anat. Record* **106**, 15.

Buchsbaum, R., and Zirkle, R. E. 1949. *Proc. Soc. Exptl. Biol. Med.* **72**, 27.

Cahan, W. G., Woodard, H. Q., Higginbotham, N. L., Stewart, F. W., and Coley, B. L. 1948. *Cancer* **1**, 3.

Cloudman, A. M., and Hamilton, K. 1949. Unpublished data.

Cloudman, A. M., Steiner, P. E., Passonneau, J. V., and Brues, A. M. 1952. Unpublished data.

Crabtree, H. G., and Cramer, W. 1932. *Proc. Roy. Soc. (London)* **B113**, 126.

Devik, F., Elson, L. A., Koller, P. C., and Lamerton, L. F. 1950. *Brit. J. Cancer* **4**, 298.

* Information regarding the availability of these reports may be obtained by addressing requests to the Office of Technical Services, Department of Commerce, Washington, D.C.

Dunlap, C. E., Aub, J. C., Evans, R. D., and Harris, R. S. 1944. *Am. J. Pathol.* **20,** 1.
Ellis, J. T., Toolan, H. W., and Kidd, J. G. 1950. *Federation Proc.* **9,** 329.
Elson, L. A., and Lamerton, L. F. 1949. *Brit. J. Cancer* **3,** 414.
Ely, J. O., and Ross, M. H. 1949. *Anat. Record* **104,** 113.
Evans, R. D., and Goodman, C. 1940. *J. Ind. Hyg. Toxicol.* **22,** 89.
Failla, G. 1940. *Am. J. Roentgenol.* **44,** 649.
Figge, F. H. J. 1947. *Science* **105,** 323.
Fournier, R. 1935. *Rev. franç. gynécol.* **30,** 445.
Friedman, M., and Hall, J. W. 1950. *Radiology* **55,** 848.
Furth, J. 1946. *J. Gerontol.* **1,** 46.
Furth, J., and Butterworth, J. S. 1936. *Am. J. Cancer* **28,** 65.
Furth, J., and Sobel, H. 1947. *J. Natl. Cancer Inst.* **8,** 7.
George, E. P., George, H., Booth, J., and Horning, B. S. 1949. *Nature* **164,** 1044.
Glücksmann, A. 1946. *Brit. Med. Bull.* **4,** 26.
Glücksmann, A. 1948. *Brit. J. Radiology* **21,** 559.
Glücksmann, A. 1951. *J. Pathol. Bacteriol.* **63,** 176.
Glücksmann, A. 1952. *Brit. J. Radiology* **25,** 38.
Glücksmann, A., and Spear, F. G. 1949. *Brit. J. Radiology* **12,** 140.
Grynkraut, B., and Flaks, J. 1938. *Bull. assoc. franç. étude cancer* **27,** 268.
Hall, B. V. 1952. Unpublished data.
Hall, B. V., Hamilton, K., and Brues, A. M. 1952. *Cancer Research* **12,** 268.
Hall, J. W., and Friedman, M. 1948. *Radiology* **50,** 318.
Harrington, N. J., and Koza, R. W. 1951. *Biol. Bull.* **101,** 138.
Hartwell, J. L. 1951. Survey of Compounds Which Have Been Tested for Carcinogenic. Activity, Federal Security Agency, U.S. Public Health Service, Washington, D.C.
Hesse, O. 1911. *Fortschr. Gebiete Röngenstrahlen* **42,** 82.
Hevesy, G. C. 1951. *J. Chem. Soc.* p. 1618.
Hueper, W. C. 1942. Occupational Tumors and Allied Diseases. Charles C Thomas, Springfield, Ill.
Jolles, B. 1949. *Brit. J. Cancer* **3,** 27.
Jolles, B., and Koller, P. C. 1950. *Brit. J. Cancer* **4,** 77.
Kaplan, H. S. 1948a. *J. Natl. Cancer Inst.* **8,** 191.
Kaplan, H. S. 1948b. *J. Natl. Cancer Inst.* **9,** 55.
Kaplan, H. S. 1949. *J. Natl. Cancer Inst.* **10,** 267.
Kaplan, H. S. 1951. *J. Natl. Cancer Inst.* **11,** 261.
Kidd, J. G. 1950. *Proc. Chicago Inst. Med.* **18,** 50.
Kirschbaum, A., and Mixer, H. W. 1947. *J. Lab. Clin. Med.* **32,** 720.
Koletsky, S., Bonte, F. J., and Friedell, H. L. 1950. *Cancer Research* **10,** 129.
Lasnitski, I. 1945. *Brit. J. Radiology* **18,** 214.
Lasnitski, I. 1947. *Brit. J. Radiology* **20,** 240.
Lea, D. E. 1947. Actions of Radiations on Living Cells. Cambridge University Press, London.
Lick, L., Kirschbaum, A., and Mixer, H. W. 1949. *Cancer Research* **9,** 532.
Lisco, H., Brues, A. M., Finkel, M. P., and Grundhauser, W. 1947a. *Cancer Research* **7,** 721.
Lisco, H., Finkel, M. P., and Brues, A. M. 1947b. *Radiology* **49,** 361.
Lisco, H., and Finkel, M. P. 1949. *Federation Proc.* **8,** 360.
Lorenz, E. 1950. *Am. J. Roentgenol.* **63,** 176.
McDonald, E., and Staff of the Biochemical Research Foundation. 1947. Neutron Effects on Animals. Williams and Wilkins Co., Baltimore.

March, H. C. 1947. *J. Am. Med. Assoc.* **135,** 179.
Marinelli, L. D., and Brues, A. M. 1953. *In* Physiopathology of Cancer, F. Homburger, and W. H. Fishman (eds.). Paul B. Hoeber, Inc., New York.
Marinelli, L. D., Norris, W. P., Hasterlik, R. J., Looney, W. B., and Brues, A. M. 1952. Unpublished data.
Marshak, A. 1942. *Radiology* **39,** 621.
Martland, H. S., and Humphries, R. E. 1929. *Arch. Pathol.* **7,** 406.
Melnick, P. J., and Bachem, A. 1937. *Arch. Pathol.* **23,** 757.
Miller, E. C. 1950. *Cancer Research* **10,** 232.
Miller, E. C., and Miller, J. A. 1947. *Cancer Research* **7,** 468.
Murphy, J. B., and Sturm, E. 1925. *J. Exptl. Med.* **42,** 155.
Neel, J. V., and Falls, H. F. 1951. *Science* **114,** 419.
Passonneau, J., Hamilton, K., and Brues, A. M. 1953. *Cancer Research.* (In press.)
Paterson, E. 1942. *Brit. J. Radiology* **15,** 177.
Petrov, N., and Krotkin, N. 1932. *Z. Krebsforsch.* **38,** 249.
Pullinger, B. D. 1932. *J. Pathol. Bacteriol.* **35,** 527.
Raper, J. R., Henshaw, P. S., and Snider, R. S. 1951. *In* Biological Effects of External Beta Radiation, p. 200, R. E. Zirkle (ed.). McGraw-Hill Book Co., New York.
Sabin, F. R., Doan, C. A., and Forkner, C. E. 1932. *J. Exptl. Med.* **56,** 267.
Sacher, G. A., Sackis, J., and Brues, A. M. 1949. *Cancer Research* **9,** 620.
Salter, W. T. 1948. *Occupational Med.* **5,** 441.
Sparrow, A. H. 1948. *Nature* **162,** 651.
Tahmisian, T. N. 1949. *J. Exptl. Zool.* **112,** 449.
Tahmisian, T. N., and Adamson, D. M. 1951. *Proc. Soc. Exptl. Biol. Med.* **78,** 597.
Tansley, K., and Wilson, C. W. 1947. *Radiology* **49,** 62.
Warren, S., Holt, M. W., and Sommers, S. C. 1951. *Proc. Soc. Exptl. Biol. Med.* **77,** 288.
Windholz, F. 1947. *Radiology* **48,** 398.
Wood, C. A. P. 1949. *J. Am. Med. Assoc.* **120,** 513.

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Survival and Preservation of Tumors in the Frozen State

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CONTENTS

I. Introduction

Although Michaelis (1905) and Ehrlich (1907) found that tumors could be transmitted with tissues subjected to freezing, it was not until 1938 that storage of tumors in the frozen state was proposed as a feasible method of preservation (Breedis and Furth, 1938). Even now, low-temperature preservation of experimental tumors is employed in relatively few laboratories as an alternative to maintenance by serial transplantation.

Much of the work reported in the literature on the transmission of tumors with frozen material has been devoted to the relative merits of slow or fast freezing or to the demonstration of cell survival. The methods that have been employed by different authors have varied so much that it is difficult to compare their results. Reports on long-term storage are few. It is therefore not surprising that, notwithstanding the obvious advantages of low-temperature preservation as an alternative to the

serial transplantation of tumors, there has been a reluctance to employ this method. If applied empirically, the freezing of tumors can yield only variable and suboptimal results. However, progress has recently been made in elucidating the phenomena which determine the survival of some tumor cells in the frozen state. Other observations on the survival of spermatozoa and erythrocytes confirm the importance of methods of "preconditioning" cells prior to freezing and indicate a considerable field for further investigation.

Some of the more malignant transplantable animal tumors may be preserved in the frozen state for years without any demonstrable loss of activity. As far as such tumors are concerned, satisfactory methods of preparation for freezing and low-temperature storage have been developed. These are based on observations of and correlations between cell state and resistance which seem to indicate the way to the development of more rational and effective methods of preservation. It remains for future investigation to determine if such methods will be applicable, with suitable modifications, to all tumors.

II. Historical Review

1. Early Experiments (1905–1937)

The earliest report indicating that tumor cells might survive freezing and thawing is that of Michaelis (1905), who stated, very briefly, that he had successfully transplanted Jensen's carcinoma after it had been exposed to liquid air for 30 minutes. In 1906 Ehrlich (1907), discussing the temperature limits of tumor survival, confirmed the upper limits reported by Jensen and Loeb but stated that the lower limit (5 minutes at −18°C.) must be reduced considerably. Ehrlich said that he had repeatedly seen tumors develop after being kept for 48 hours at −25° to −30°C. Furthermore, he had even obtained a tumor with a carcinoma that had been stored continuously in the refrigerator for 2 years at −8° to −10°C., and the limit had not been reached in the preservation of a chondroma. Apolant (1914) confirmed that Ehrlich obtained one tumor with the carcinoma stored for 2 years but added that he also obtained sixty negative results.* Salvin-Moore and Walker (1908) and Salvin-Moore and Barratt (1908) exposed fragments of an Ehrlich mouse tumor and a Jensen mouse tumor, respectively, to liquid air for 20 to 30 minutes and then grafted these fragments subcutaneously. Some tumors were obtained, but the percentage of successful grafts is not stated. Although these authors pointed out the alternatives of cell sur-

* "Ja in einem Falle ging allerdings nur eine von 60 Impfungen mit einem Karzinommaterial an, das volle 2 Jahre bei −8–10° aufbewahrt worden war." (1914, p. 367.)

vival and causal virus and referred to the survival of plant seeds and trypanosomes at −125°C., they refrained from drawing conclusions from their observations.

Gaylord (1908) repeated these experiments of Salvin-Moore and his collaborators, using a mouse carcinoma. He obtained two tumors in nine mice with a portion which was frozen for 40 minutes, three tumors in fourteen mice with a portion frozen for 80 minutes, and tumors in all of five mice with the control portion. In some instances the mice were killed in 4 to 7 days and the graft examined for growing cells. This author also found that embryonic mouse tissue was killed by freezing with liquid air but that *Trypanosoma gambiense* survived for 20 minutes, although not for 40 minutes.

Cramer (1930) described more detailed observations on the effect of freezing on a series of transplantable tumors. He employed Jensen rat sarcoma, S 37, Crocker sarcoma, and carcinomas 63, 91, and 113. These tumors were finely minced and subjected to repeated freezing and thawing, (a) four times to below −20°C. on a freezing microtome or (b) four to eight times to −80°C. using liquid air, and thereafter tested by inoculation in a large number of experiments. Cramer reported the results of eighty-two experiments utilizing 695 mice. In addition, attempts to obtain tissue culture growth with S 37 and carcinoma 63 were made, but these gave negative results. The treated carcinomas were found to be consistently inactive, but tumors were obtained in twenty-four out of fifty experiments with the sarcomas, and these were found to be histologically similar to the parent tumor. A greater number of tumors was obtained with S 37 frozen to below −80°C. Further experiments showed that the transmitting property was evanescent, being removed by washing with saline and also disappearing rapidly on incubation. Cramer stressed the differences exhibited "even by cells of one and the same sarcoma strain—37 S—when tested at different times." He drew no definite conclusions but considered three possibilities: (a) that sarcoma cells survived freezing but that the resistance to freezing varied greatly at different times; (b) that mammalian malignant neoplasms might be transmitted without the intervention of cells; and (c) that cells damaged but not disintegrated by freezing could reconstitute their structural organization and "resume life."

Auler (1932) carried out experiments with the Ehrlich carcinoma, the Jensen rat sarcoma, and the Flexner-Jobling tumor. The tumors were ground up in a mortar, diluted with 5 to 10 parts of saline, and frozen by means of CO_2 ice for 5 to 35 minutes. The Flexner-Jobling tumor was found to be inactive after this treatment, but some tumors were obtained with the others. These were histologically similar in type to the mother

tumor, and Auler concluded that cells must have survived and that the presence of a carcinogenic "Zellkolloide" could be ruled out. Klinke (1937) reported a series of experiments in which the Ehrlich mouse sarcoma was frozen in liquid nitrogen. Tumors were obtained not only with suspensions subjected to brief freezing (3 minutes) but also with frozen tumor subsequently stored at −20°C. for 2 weeks or for 47 hours in an icebox.

No useful purpose would be served by proceeding with a chronological summary of the literature beyond this point, for subsequent authors deal with various aspects of the subject which are best discussed separately. However, the next important contributions should be briefly mentioned here. Barnes and Furth (1937) and Breedis *et al.* (1937) found that "the transmitting agent of the leukemia of mice, presumably malignant leucocytes," previously considered to be inactivated by freezing, would remain viable even at −70°C. if frozen slowly. Breedis and Furth (1938) reported observations on tumors which had been kept in the frozen state up to 448 days. Klinke (1939, 1940) was successful in obtaining growth in tissue culture from tumors frozen at −196°C., thus providing direct proof of cell survival under these conditions.

2. Influence of Rate of Freezing

Although various workers have investigated the effects of rapid and slow freezing and thawing on tumor survival, it is difficult to compare the results because of differences in materials and techniques employed. The terms "rapid" and "slow" have been used in a relative sense, and the fastest rates of freezing employed are insufficient to prevent crystallization of water and produce the intracellular vitreous state which Luyet and Gehenio (1940) have contended is essential to cell survival. According to the vitrification hypothesis of these authors, it is essential to cool and also to thaw the cell or organism so rapidly that amorphous solidification will occur before ice crystals can form. Cooling at the rate of at least several hundred degrees per second to a temperature below −40°C. (approx.) is required. Because the rate of heat transfer is the limiting factor, this ultrarapid cooling cannot be achieved if the thickness of the preparation exceeds 0.1 mm. and the water content exceeds 70% (Luyet, 1951). Organisms which can be subjected to vitrification and equally rapid thawing may survive this treatment, but they are killed by slow rates of freezing and thawing which are as fast as the fastest rate investigated in tumor survival studies. With most tumors slow freezing combined with rapid thawing has given better survival than rapid freezing, and slow thawing is deleterious. It is evident, therefore, that the survival of tumors in the frozen state cannot be explained by any modification of the vitrification hypothesis.

It has been mentioned that Barnes and Furth (1937) were the first to observe the importance of slow cooling. These authors, together with Breedis *et al.* (1937), found that leukemic cell preparations cooled rapidly to −37°C. and held at this temperature for 30 minutes failed to produce leukemia, but that if the preparations were cooled slowly to −70°C. they retained the ability to transmit the disease when held in the frozen state for periods up to 32 days. Breedis and Furth (1938) reported observations on a number of mouse tumors subjected to slow freezing under the title "The Feasibility of Preserving Neoplastic Cells in the Frozen State." Breedis and Furth minced the tumors with scissors into a small amount of Tyrode solution on an iced plate. The suspension was sealed in glass tubes which were placed in an alcohol bath and slowly cooled by the addition of fragments of CO_2 ice. After freezing the material was stored on CO_2 ice, and when required for test was thawed quickly because this was thought to be less injurious than slow thawing. Excellent survival was obtained with the following tumors: lymphocytic leukemia (survival for 440 days), myelocytic leukemia (440 days), monocytic leukemia (430 days), sarcoma 3172 (448 days), and mammary carcinoma (98 days). A strain of chloroleukemia was found difficult to preserve, but it survived if large fragments of spleen were frozen. Breedis and Furth also noted that chicken tracheal epithelium kept in the frozen state for 327 days exhibited ciliary motion on thawing. They concluded that the tumor inducing activity of the frozen-preserved materials is attributable to the survival of living cells—not virus—because frozen tumor suspension irradiated at −70°C. with 4000 r (a dose of x-rays sufficient to kill cells but not viruses) was completely inactivated.

Mider and Morton (1939) compared the effects of two rates of freezing on solid portions of S 37, S 180, and the Walker rat carcinoma. Rapid freezing was carried out by immersing the tube containing the tissue in a bath of Methyl Cellosolve and CO_2 ice at −74°C. A thermocouple inserted into the tissue showed that 3 to 5 minutes were required to reach the temperature of the refrigerant. Slow freezing was effected by starting with the bath of Methyl Cellosolve at room temperature and cooling it by the addition of CO_2 ice fragments at a rate sufficient to give the minimum temperature in not less than 20 minutes. The frozen tissue was thawed at room temperature or in a water bath at 30°C. and cut into fragments. These were grafted subcutaneously in the inguinal region on one side, a control (unfrozen) portion of tumor being grafted on the other. Mider and Morton's results, unfortunately, are given only in percentages of grafts which grew to form tumors. Tests were carried out with tumor frozen and thawed up to six times, presumably with the idea of enhancing any differences due to rate of freezing. In all instances where tumors developed the latent period was prolonged with frozen and thawed

material. When the tumor tissue was frozen once only, no significant difference was attributable to the rate of freezing; 8 to 90% of sarcoma grafts and 45 to 50% of the carcinoma grafts were successful. The results obtained with repeated freezing and thawing might seem to suggest that rapid freezing gave better survival. However, the data are incomplete, and it is doubtful whether they can be accepted at their apparent face value in view of the fact that many grafts failed to take. It is to be noted also that Mider and Morton kept the tumor tissue frozen for periods ranging from 5 minutes to 24 hours before thawing, and these variations may have influenced the results. These authors also tested the effect of freezing on tumor suspensions prepared in 3 parts of buffered Ringer. The sarcoma suspensions did not survive freezing and thawing in this state, but some tumors were obtained with the carcinoma suspension when it was frozen slowly. Klinke (1940), who succeeded in obtaining growth in tissue culture from fragments of carcinomas and sarcomas, emphasized the need for rapid freezing and thawing.

Breedis (1942) made a careful and detailed analysis of the effect of the rate of freezing on leukemic cells, and his results clearly show slow freezing to be less destructive than fast freezing. Breedis stated that he found this difficult to explain because profound changes may occur when materials are kept frozen for a protracted time close to the freezing point; for example, Moran (1929) found that the freezing of frog muscle at −2°C. to equilibrium removed 78% of the water as ice.

Breedis' methods and results merit detailed consideration. He prepared the leukemic cell suspensions by mincing tumor, spleen, and lymph nodes in Tyrode solution with 10% rabbit serum or amniotic fluid and took the important precaution of filtering the suspension through cotton to remove cell clumps. The temperature changes which occurred during slow freezing were recorded by thermocouples inserted into specially designed tubes containing the suspensions. Flat tubes with very thin walls were used for the study of the effect of rapid cooling through various temperature ranges. Three strains of leukemia were compared in regard to their resistance to rapid or slow freezing combined with rapid or slow thawing. Suspensions to be frozen slowly were placed in thin-walled tubes in an alcohol bath at 0°C., and the temperature of the bath was lowered by approximately 0.5° per minute by adding small pieces of solid CO_2. At −60°C. the rate was increased to 1.0° per minute, and at −70°C. the tubes were immersed in liquid nitrogen at −196°C. Rapid freezing was accomplished by allowing the suspension to fall drop by drop onto different parts of the inner wall of a thin-walled tube immersed in liquid nitrogen. All suspensions were kept at −196°C. for approximately 1 hour and were thawed as needed for injection. For rapid thawing the tube was

transferred to alcohol at −40°C. for 5 minutes and then shaken in water at 37°C. Slow thawing was effected by placing the tube in a small beaker containing alcohol at −43°C. in the icebox. A tube containing the original suspension was kept in ice water until the other tubes had been frozen, and this suspension was then titrated by inoculating a series of dilutions made in Tyrode solution to which 10% rabbit serum or amniotic fluid had been added. The frozen and thawed suspensions were injected without dilution. The average length of life of the mice after inoculation with dilutions of the control suspension gave a fair indication of the concentration of the transmitting agent, and from these data an approximate estimate of the survival values of the frozen leukemic cell suspensions could be obtained. With one exception, where the material was slow-frozen and slow-thawed, survival was demonstrated only in suspensions subjected to slow freezing and rapid thawing. The least resistant strain of leukemia showed a survival of only 1 in 10,000 to 1,000,000.

Breedis then proceeded to a detailed analysis of the effect of rapid cooling through various temperature ranges using one of the more resistant strains of leukemia. A large number of preparations were subjected to different rates of cooling through different parts of the temperature range from 0° to −70°C.; when this temperature was reached, each preparation was cooled to −196°C. Breedis' observations show quite clearly that the "changes which are peculiar to rapid freezing alone and lead to complete inactivation take place during rapid transition from the liquid to the solid state, in a range of temperature lying between −15°C. and the freezing point." He remarked that "the sharp end point at which the cooling rate causes complete inactivation is remarkable. Approximately 1 per cent of activity was preserved whether cooling through the range 0° to −15°C. required 30 minutes or 1 minute, but when this range was passed through in 12 seconds or less, the material became innocuous, its activity being reduced to less than 0.0001 per cent."

In the article in which these observations are presented Breedis reviewed the literature and discussed the possible mechanisms of death by freezing. He stated: "Results that show slow freezing to be less destructive than rapid freezing are difficult to explain," but, arguing from the observations of Moran (1926) (see Section III.1), he considered that the dehydrating effect of freezing, leaving protoplasm a less favorable site for ice crystal formation provides a likely explanation.

Snell and Cloudman (1943) investigated the rate of freezing on the survival of fourteen transplantable tumors in mice. Thin slices of tissue (× 2 × 6 mm., approximately) were placed in vials containing Freon 11 or isopentane, either at −79°C. (fast freezing) or at room temperature. For slow freezing the vial, or alternatively a small piece of tissue, inserted

into a dry tube, was placed in CO_2 ice. The following tumors were employed: three lymphoid leukemias, two myeloid leukemic tumors, a teratoma, a melanoma, a reticuloendothelioma, four-mammary carcinomas, and two fibrosarcomas. The teratoma and some of the leukemias did not stand freezing, and the others showed different degrees of survival. The results, judged from the percentage of tumors which appeared and their average lag, show trends which suggest that rapid freezing produced more severe damage.

Excluding the contribution of Breedis concerning leukemic cells, it is impossible to draw any firm conclusions regarding the relative merits of fast and slow freezing from the literature which has been cited. It is probable that the results obtained by various workers have been influenced by numerous factors introduced by variations in technique and choice of different tumors. Comparisons of survival have been hampered by the lack of suitable quantitative methods applicable to solid tumors. The careful observations of Breedis (1942) might appear to prove beyond question the superiority of slow freezing, especially in the range of 0° to −15°C. However, an unexpected factor discovered by Gabrielson *et al.* (1952) might seem to invalidate the work of Breedis. Gabrielson *et al.*, using other strains of leukemia, found that, although fast-frozen suspensions appeared inactive when stored for 24 hours at −76°C., activity reappeared on further storage at this temperature. Accordingly, they postulate a labile inhibitory factor to explain this recovery of ability to transmit leukemia. Undoubtedly there is evidence that elusive labile factors may reduce the activity of freshly frozen tumor suspensions (Craigie, unpublished), but the observations of Gabrielson *et al.* do not prove that Breedis' results were due to failure to keep his material frozen for 72 hours before testing, for, here again, we are faced with the difficulty of comparing and interpreting results of different observers. Gabrielson *et al.* used unfiltered spleen mash suspensions, they did not freeze as quickly as Breedis, and they did not investigate the 0° to −15°C. temperature range.

3. Preservation in Dextrose Solutions

Much of the work which has been cited was carried out with tumor tissue frozen *en masse*. Breedis and Furth (1938), however, used tissue finely minced with scissors in a small volume of Tyrode solution, and Breedis (1942) used leukemic cell suspensions containing 10% normal rabbit serum or amniotic fluid. Mider and Morton (1939) tested saline suspensions of the Walker rat carcinoma but found that these were more readily inactivated by freezing than solid portions of this tumor. Craigie (1949b), with a view to utilizing low-temperature preservation of tumors

in the development of more quantitative methods for the study of transplantable tumors, investigated the survival of tumor cell suspensions when subjected to freezing in a variety of fluids. He considered that "it might be expected that cells exposed to an artificial environment are more likely to be killed by freezing than are cells situated in the interior of a piece of intact tissue, because the former are more exposed to the mechanical effects of ice formation and to the strong concentrations of inorganic salts induced between the initial freezing point and the freezing temperature of their eutectic solutions." Preliminary experiments showed that the rate of inactivation referable to electrolyte concentration was undesirably high, and Craigie therefore investigated the suitability of dextrose solutions as suspending fluids. He found that dilute cell suspensions prepared from a C_3H sarcoma would survive freezing and thawing in 5.3% dextrose solution. The protective effect of dextrose was evident when used in concentrations ranging from 3 to 40%, the optimum being between 5 and 7%. A C_3H sarcoma suspension diluted 1 in 12.5 and frozen in 10% dextrose solution containing 40% glycerol was found to produce tumors in all of twenty-four inoculated mice after storage for 253 days in the frozen state on CO_2 ice. Calculations from the mean lag period for two dilutions tested indicated that probably 25% of the tumor cells survived freezing and thawing. In presenting these results, Craigie did not discuss any alternative to cell survival.

In view of the evidence of survival obtained on freezing in hypertonic dextrose and glycerol solutions Craigie (1949c) began an investigation of the effect of dehydration. A number of C_3H sarcoma suspensions were dried from the frozen state in dextrose solution. On one occasion tumors were obtained with the dried material, and Begg subsequently confirmed this. These observations, together with others on the activity of frozen-dried material, were reported by Gye (1949) and Gye *et al.* (1949).

4. Demonstration of Cell Survival

Gye interpreted the observations of Craigie and Begg on the transmission of tumors with tissue frozen-dried in dextrose as evidence that the continuing cause of the tumors is probably a virus, although Craigie, on the basis of quantitative estimates (1949c and 1950), considered that the survival of one cell in a million was sufficient to account for the positive results obtained. At this time there was little direct evidence available concerning the ability of tumors or normal somatic cells of homotherms to withstand freezing. Auler (1932) had noted that tumors induced by inoculation with Ehrlich mouse carcinoma and Jensen rat sarcoma after freezing were similar histologically to the mother tumor, and he pointed out that this indicated cell survival. Mider and Morton (1939) grafted

rat skin which had been subjected to freezing. They found that squamous epithelial and connective tissue cells grew after a single freezing to −74°C. Rapid freezing produced more cellular damage than slow freezing. Klinke (1939, 1940) claimed growth in tissue cultures with Jensen rat sarcoma which had been kept at −196°C. for 2 days and with Ehrlich mouse sarcoma after it had been frozen for 10 minutes at −253°C. Webster (1944) described successful takes with human skin grafts refrigerated at −72°C. Briggs and Jund (1944) demonstrated that mouse skin remains viable after slow freezing on CO_2 ice and rapid thawing. Grafts of ventral skin from young mice were kept in a frozen state from 1 to 48 hours and then were grafted autoplastically to the dorsum; 52% of these grafts took wholly or in part and persisted as functional skin. Strumia and Hodge (1945) successfully transplanted autogenous split-thickness grafts of human skin which had been preserved in the frozen state from 1 to 61 days at temperatures of −20° to −25°C. In three patients, 80.5% permanent takes were obtained with forty-one frozen grafts and 86.4% takes with thirty-four control grafts.

Gye and Mann's interpretation of observations on the transmission of tumors with frozen or frozen-dried materials (Gye *et al.*, 1949; Mann and Dunn, 1949) had the fortunate effect of stimulating further investigation. Passey and Dmochowski (1950) found that when suspensions of minced tumor tissues which had been previously frozen, or frozen and dried, were centrifuged, inoculation of the supernatant failed to produce tumors, whereas the cell deposit was active in this respect. Passey *et al.* (1950) demonstrated sarcoma cell survival by applying tissue culture methods to suspensions of cells which had been frozen in glucose solutions and desiccated. Histological evidence that the propagation of tumors with frozen-dried tissues is due to cellular transmission was obtained by Dmochowski and Millard (1950), who used the technique of subcutaneous grafting in plasma clot (Des Ligneris, 1930). Warner *et al.* (1950) investigated histologically the fate of grafts of S 37 mince after exposure to low temperatures and freeze-drying. The grafts were removed 3 hours to 10 days after implantation. In the earliest stages extensive necrosis was observed, but in 24 hours characteristic "T" cells appeared at the periphery. The number of "T" cells was roughly proportional to tumor-producing activity, and they were presumed to be tumor cells which had migrated out from the inoculated sarcoma mince.

Blumenthal and Walsh (1950) autotransplanted thyroid gland of guinea pig which had been frozen at −70° or −190°C. One out of twelve autotransplants was successful with gland frozen at −70°C., and, in addition, one parathyroid transplant was obtained. When the tissue was frozen by immersion in liquid nitrogen, eight out of twelve autotrans-

plants were successful. Kreyberg and Hansen (1950) obtained successful autotransplants of mouse ear epithelium frozen *in situ* and then transplanted immediately. Serial short-interval studies of subcutaneous transplants of S 37 mash subjected to freezing were made by Walsh *et al.* (1950). In 4 to 6 hours all the material had become necrotic, but a few surviving cells were found on the surface of the abdominal muscle. These tumor cells began to proliferate in 48 hours. Walsh *et al.* concluded that in general freezing does not appreciably alter the latent period of tumor transplants or their morphological characteristics. Ludwin (1951) showed that two mouse adenocarcinomas survived freezing with CO_2 ice, by inoculating the thawed tumor mash, mixed with charcoal as a locating agent, into 5-day embryonated eggs. Tumor growths were found in a number of eggs surviving to the eighteenth day, and these showed mitosis and the histology of the original tumor.

Transplantation experiments which demonstrate in yet another way that living tumor cells survive freezing were carried out by Bittner and Imagawa (1950) and by Law (1951). Bittner and Imagawa showed that spontaneous mammary tumors frozen in dextrose could be transmitted only to mice of the same genetic constitution as the primary host. All the strains of mice used for testing were susceptible to the milk agent. A tumor which arose in an $A/C_3H/F_1$ breeder was frozen for 48 hours. This produced tumors in $C_3Hb/Ax/F_1$ mice but not in C_3H, C_3Hb, A, or Ax. A C_3H carcinoma (thirty-third passage) was tested after being kept at $-79°C.$ for 17 days. Tumors were obtained in C_3H, C_3Hb, and $Ax/C_3Hb/F_1$ mice but not in A or D.

Law employed two mammary tumors of C_3H mice, one containing the milk agent, the other not. These were frozen at $-79°C.$ and after thawing were transplanted to C_3Hb and $RIL/C_3Hb/F_1$ hybrid mice. Progressively growing tumors were obtained. These were transplanted for five serial transfer generations in $RIL/C_3Hb/F_1$ hybrid mice and then tested in C_3Hb, RIL, and F_1 hybrids. The tumors grew in F_1 hybrids and C_3Hb but not in the RIL strain.

III. Resistance of Tumor Cells to Freezing and Thawing

1. General Considerations

The extensive literature pertaining to survival or death of organisms at freezing temperatures has been reviewed by Luyet and Gehenio (1938, 1940). Much of this literature is irrelevant, for it is concerned with organismal or systemic death whereas cell survival depends on a number of intrinsic and extrinsic factors which prevent cellular or protoplasmic death. The distinction between cellular and cytoplasmic death is not an

entirely satisfactory one, but it serves to emphasize that cell death during freezing or thawing or in the frozen state may be brought about in a number of different ways. Cellular death occurs when the traumatic effects of freezing and thawing produce gross cytological changes which appear to be incompatible with survival. Protoplasmic death is to be inferred in those instances where quick freezing of a tissue gives excellent morphological preservation but the cells do not remain viable (*vide* Parkes and Smith, 1953), and their death must be attributed to unknown physicochemical changes due to internal freezing.

There are three ways in which tumor cells may be grossly damaged during freezing: (1) by pressure and shear, (2) by penetration of the cell membrane by ice crystals, and (3) by exposure to hypertonic concentrations of salts present in the intercellular fluid. It is to be expected that the relative importance of each of these effects will vary according to the type of tumor and the way in which the tumor tissue is prepared for freezing.

1. Pressure and shear effects caused by the expansion of ice and the rapid flow of fluid between the crystals are possibly of greatest importance in coherent tumor tissue frozen *en masse*. However, it should be borne in mind that the same disruptive forces are applied when a tumor is finely minced in order to prepare a cell suspension. This effect is well shown with the pressure mincer designed by Craigie (1949a). This instrument, when a close-fitting and finely grooved plunger is used, effectively strips the cytoplasm from many kinds of normal and neoplastic cells, e.g., liver or differentiated mammary carcinoma cells. This selective effect aids in the preparation of suspensions in which the surviving cells are those that are relatively more resistant to subsequent manipulations.

2. Although it must be accepted that cells may be killed as a result of penetration by the sharp growing points of ice crystals, it is probable that this is the least important cause of death when tumor tissue or cell suspensions are subjected to freezing. Should penetration occur, death is to be attributed to consequent intracellular ice crystal formation and not to injury to the plasma membrane *per se*.

3. During freezing, the separation of ice crystals above the eutectic temperatures of the salts in solution results in the development of strongly hypertonic solutions. Consider the figures for salts present in Ringer solution: the eutectic mixture of KCl with water is 25%, and its freezing point is −10.7°C.; of NaCl, 30.4%, with a freezing point of −21.2°C.; of $CaCl_2$, 48%, with a freezing point of −52°C. This means, for example, that if a cell suspension is frozen at a temperature above −20°C. in 34 volumes of 0.85% sodium chloride, the volume of concentrated salt solution will be approximately equal to that of the cell and its concentration will be 30%. Under such conditions cell death is to be

expected. On the other hand, if the volume of extracellular fluid is small in relation to cell volume as in solid tumor frozen *en masse*, or, alternatively, if a cell suspension is diluted with a nonelectrolyte such as dextrose or glycerol, cell survival may be expected, provided that the temperature is not lowered too quickly and that the cells are able to withstand a certain degree of dehydration by exosmosis. Luyet and Gehenio (1940) classified organisms, spores, and seeds which survive at low temperatures (in this instance, at the temperature of liquid air) into three groups: (1) those that survive when dry, (2) those that survive when wet, and (3) those that survive ultrarapid cooling and thawing only while wet and provided that some degree of plasmolysis has been induced by exposure in hypertonic sodium chloride, sucrose, or glycerol solution.

It has long been known that, when tissue is frozen, water migrates from the cells and passes into the intercellular spaces. Moran's observations (1926) on gelatin gels demonstrated that the presence of electrolyte is not an essential factor in this migration of water, although the process in tissues may well be accelerated by the presence of intercellular electrolyte. Cells may be killed if plasmolysis proceeds too far, but, on the other hand, partial dehydration may promote survival. Breedis (1942) considered that the dehydrating effect of slow freezing, leaving the protoplasm a less favorable site for ice crystal formation, is a probable explanation of the survival of leukemic cells exposed to freezing and thawing, provided that the temperature is not lowered too quickly through the range 0° to −15°C. In this connection Breedis quoted the observations of Moran on gelatin gels. Moran (1926) found that ice crystals do not form in gelatin gels containing less than 35% water. If discs of higher gelatin content than 12% are frozen slowly, ice forms only on the outside of the discs until a final equilibrium of 54.3% at −3°C. or 65.2% at −19°C. is attained. Thereafter ice does not form within the gel even after immersion in liquid air. On the other hand, after rapid freezing, numerous ice foci form throughout the discs. Luyet and Thoennes (1938) observed that survival of onion epidermis was promoted if previously dehydrated by plasmolysis in hypertonic salt solution; Luyet and Hodapp (1938) found that a large percentage of frog spermatozoa showed motility after being partially dehydrated in 2 *M* sucrose, frozen in a thin film of liquid air, and thawed rapidly at 20°C. The view that partial dehydration of certain types of cell causes increased resistance to freezing is further supported by more recent observations, e.g., on the remarkable protective effect of glycerol on fowl spermatozoa (Polge *et al.*, 1949).

Dehydration may occur during freezing and presumably may be brought about in two ways: (1) directly by extracellular ice formation, as in Moran's gelatin gel experiments, and (2) by exosmosis due to the

formation of hypertonic salt concentrations above the eutectic points (Table I).

TABLE I
Effects on Cell Survival of Physical Changes during Freezing

Physical Change	Physical Effects on Cell	Cell Survival
Ice formation	(a) Pressure and shear	Prejudiced
	(b) Dehydration	Favored or prejudiced
Increased extracellular salt concentration	(a) Injury to plasma membrane	Prejudiced
	(b) Dehydration	Favored or prejudiced

It is to be expected from a consideration of the physical changes which occur during freezing and the possible effects of these on the cell (Table I):

1. That survival of the cell up to the point when intracellular freezing may occur depends on its ability to withstand (a) pressure and shear, (b) exposure to hypertonic salt solution, and (c) the dehydrating effects of extracellular ice formation and of exosmosis.

2. That different types of tumor may differ greatly in their ability to survive when frozen *en masse*.

3. That survival may vary according to rate of freezing.

4. That the composition of the suspending fluid may have a profound influence on survival of tumor mince or cell suspensions.

In the absence of evidence to the contrary it seems reasonable to assume that, unless freezing in individual cells is ultrarapid, the changes induced by intracellular ice formation may cause death by disrupting the submicroscopic organization of cytoplasm and nucleus and by producing irreversible physicochemical changes. The observations of Hazel *et al.* (1949) on the effects of freezing colloidal silicic acid are of interest in this connection. The stability of this colloidal system depends on the temperature to which it is rapidly frozen and also on the rate of thawing. If frozen at temperatures below −55°C. and thawed rapidly, the system remains stable. It also remains stable for an indefinite period if frozen in liquid air and kept at a temperature below −55°C. If, however, the frozen sol is transferred to a temperature of −35°C. for 15 minutes, it coagulates irrespective of the rate of subsequent thawing. Hazel *et al.* relate this phenomenon to the fact that at a temperature of about −55°C. a polymorphic transition occurs from a fine ice structure with a disoriented lattice (not a vitreous state) to a more orderly lattice with a higher lattice energy, this higher lattice energy being sufficient to overcome the solvation energy.

It is unknown whether cell survival on storage at temperatures from

−70° to −79°C. depends on the intracellular fluids remaining unfrozen. Luyet and Gehenio (1940) suggested that the absence of freezable water may be the important factor in the survival of some cells under certain conditions. However, it may be argued that sudden intracellular freezing from a supercooled state may result in vitrification when CO_2 ice is used as refrigerant. Luyet (1951), in a recent summary of the principles and techniques of vitrification by rapid cooling, stated that the "dangerous" crystallization temperature range for protoplasm is between zero and some tens of degrees below zero. Because rate of transfer of heat is a limiting factor, "it is impossible to reach the cooling velocity required for vitrification—which is of the order of several hundred degrees per second —with any object which measures more than about 0.1 mm. in one dimension when the water content is between 70 and 80 per cent." In order to attain the necessary rate of ultrarapid cooling, a very high temperature differential is necessary and the organisms or tissue to be cooled must be immersed in a fluid cooled in a liquified gas such as liquid nitrogen. However, it would appear that it is not necessary to cool the object to a temperature approaching −200°C. but merely to cool it with very great speed to a temperature which probably lies between −32° and −55°C. The former figure is that which might be inferred from Luyet's statement (1951), and the latter follows from the remarks of Hazel *et al.* regarding ice structure. It may therefore be suggested that, when tumor tissue or a suspension of tumor cells is frozen to a temperature of −70°C. (approx.), cells may become vitrified provided that the rate of cooling has not been too rapid. This paradoxical effect, if it does occur, would arise in the following way. Slow cooling exerts a dehydrating effect on unfrozen cells, thus depressing the freezing point of the protoplasm and favoring supercooling. If supercooling proceeds to below the transition temperature of ice (−55°C., approx.), subsequent solidification of the cell to a vitreous or semivitreous state is likely to occur with extreme rapidity. Some support for this view seems to be provided by the fact that rapid thawing of frozen tumor suspensions is essential if maximum survival is to be secured. It is difficult to understand why this should be so if the surviving cells had remained unfrozen because, in this event, a slow reversal of the freezing process would seem more likely to favor survival during thawing. On the other hand, if intracellular vitrification should occur, maximum survival is to be expected only if the cells are warmed as rapidly as possible through the dangerous temperature range of ice crystallization.

2. Microscopic Observations

A number of difficulties have impeded the investigation of tumor survival in the frozen state and the development of techniques for low-

temperature preservation. When tumors are obtained by the grafting of tumor tissue which has been frozen *en masse*, the results yield little information beyond the fact that a sufficient number of tumor cells survive to initiate growth. The surviving cells may represent a very small proportion of cells viable in the graft before freezing, and accurate quantitative comparisons of technique are impossible. Statistically significant differences in mean lag period may be of some value as an indication of gross differences in the number of tumor cells surviving. Differences in the percentages of takes, however, should be interpreted with caution, because when the proportion of viable cells in a graft is reduced below the level required to give 90% takes a number of other factors play an important part in determining whether tumor growth will become established. The most important of these are (a) the reaction of the graft to the dead tissue, (b) the ability of viable cells to migrate to the periphery of the reaction zone, and (c) adequacy of the initial stimulus to vascularization. Another factor becomes operative if tumors such as S 37, which arose in hybrid mice, are used; this is (d) the development of tumor resistance which may overtake delayed growth of tumors arising from small numbers of cells.

In some tumors, e.g., S 37, the tumor cells vary greatly in their resistance to freezing, and the distribution of resistant cells in the tumor is quite irregular. It is therefore impossible to obtain valid comparisons with portions of such a tumor subjected to different methods of freezing *en masse* (Craigie, unpublished). Consequently, useful comparisons can be made only if the tumor is first reduced to a homogeneous single-cell suspension which can be divided into aliquot parts, e.g., Breedis (1942) with leukemic cell suspensions. Unfortunately, there is no satisfactory method of reducing solid tumors to single cell suspensions without destroying a large proportion of tumor cells in the process because of their susceptibility to pressure and shear. The pressure mincer designed by Craigie (1949a) for the preparation of tumor suspensions is a useful and convenient tool for the selective destruction of tumor cells least likely to survive freezing (Section III.1).

A further difficulty arises from the fact that cells which may appear to be normal on cytological examination may be incapable of initiating tumor growth on transplantation. Craigie (Craigie *et al.*, 1951; Craigie, unpublished) has studied extensively the correlations between the capacity of free tumor cell suspensions to initiate tumors and the phase microscopy of the cells present. Initially, confusing results were obtained when control and treated tumor cell suspensions were titrated subcutaneously in serial dilutions in mice and portions of these serial dilutions were mounted on cytometer slides and recorded photographically, for

comparison with the *in vivo* tests. A variety of extracellular and intracellular physiological fluids were used, and no correlation whatever could be established between cell count and tumor-inducing activity under the experimental conditions employed. Some suspensions which on phase contrast examination appeared to consist almost entirely of intact and presumably viable cells were found to be devoid of any tumor-inducing activity, whereas others in which all cells appeared to be grossly damaged proved to be almost as active as the untreated control suspension. When dextrose solutions were used as suspending fluids, the results became even more puzzling, for dextrose produces immediate and striking changes in free tumor cells obtained by mincing techniques. The nucleus swells, and the nucleoli and associated masses of chromatin become invisible; later the cytoplasm disintegrates and separates from the cell. (This effect of dextrose cannot be attributed entirely to preliminary trauma during mincing because the same effect may be observed in a small proportion of ascites tumor cells when these are transferred to dextrose solutions.)

In these studies phase contrast objectives having a phase plate transmission of approximately 50% were used initially. It was noted that tumor cell suspensions prepared from transplantable mouse tumors which show excellent survival in the frozen state in dextrose solution (C_3H sarcoma, S 37, C_{57} sarcoma, and carcinoma 63) contained a small and variable number of extremely refractile bodies which were at first assumed to be dead cells derived from necrotic areas of the tumor. However, it finally became evident from the quantitative studies that the tumor-inducing activity of frozen and thawed cell suspensions was consistently and directly proportional to the number of "refractile" cells present. It was also observed that such cells might swell and lose their refractility when transferred to Ringer or Tyrode solution (Craigie *et al.*, 1951). Considerable quantities of "refractile" cells in a relatively pure state were obtained by growing S 37 or C_3H sarcoma as ascites tumors, treating the cellular peritoneal exudate with dextrose solution, and fractionating the cells by differential centrifugation. It was established that a very high percentage of the tumor cells which had assumed the inactive "refractive" state survived freezing and thawing in dextrose solution and that some survived drying from the frozen state. For example, a pure suspension of S 37 "refractile" cells was dried in a single cell layer in dextrose at $-20°C$. On reconstitution 0.2% of these cells were found to have retained their "refractile" appearance. Eight mice were injected with an average subcutaneous dose of 75 of these surviving cells; in five of these tumors were noted 12 days after inoculation and seven showed growing tumors by the twenty-first day (Craigie *et al.*, 1951).

When cells pass into the resistant "refractile" state in which they are

able to survive under conditions which are rapidly lethal for active cells, they shrink and assume a rounded form. This change is accompanied by a change in refractive index and, as a result, the normal phase contrast image is replaced by one which is largely optical artifact even when high transmission phase plates (T = 70 to 80%) are employed. A marked optical membrane is present at the boundary of the cell and is accompanied by brilliant internal and external halos (Fig. 1), and the nucleus of the

TABLE II

Distinguishing Characteristics of α and β Cell Phases of Diphasic Tumors

Extreme α Type	Extreme β Type
Dedifferentiated.	Differentiated.
Actively mobile.	Sedentary.
Free growth on peritoneal and pleural exudate.	Dependent upon supporting surfaces and adequate supply of O_2.
High and uniform cytoplasmic absorption at 2536 Å. Other evidence of high cytoplasmic PNA.	Low or irregular cytoplasmic absorption at 2536 Å, according to type of tumor.
Imperfect phase contrast image (see text).	Normal phase contrast image showing nuclear and cytoplasmic detail.
Relatively resistant to pressure and shear.	Sensitive to pressure; cytoplasm readily stripped from nucleus by shear.
Killed by freezing unless conditions permit change to P state.	Killed by freezing.

cell is invisible. It is a matter of very great difficulty to distinguish tumor cells from other cells (e.g., macrophages) when both are in the "refractile" state, and it is impossible to distinguish one tumor from another when only "refractile" cells are available for comparison. Craigie (1952a) proposed the term "paramorphic" to designate this resistant and inactive cell state.

In the course of investigations into the origin of these paramorphic cells and their resistance to freezing Craigie (1952a,b) found it necessary to recognize in addition two extremes of active cell states in certain diphasic experimental tumors (C_3H sarcoma, S 37, and T 2146). These active states, termed α and β phases, show the ultraviolet absorption characteristics of the extreme type A and type B cells described by Caspersson and Santesson (Caspersson, 1950). Rapid transformation from one state to the other may be induced *in vitro* by appropriate manipulations. Similar changes of state occur *in vivo*. When a diphasic tumor forms a solid vascularized growth, the majority of cells proliferate in the β phase, but when it is propagated as an ascites tumor, the cells grow in the α phase in a free state in the exudate they induce. It should be appreciated

that change of cell state occurs in response to environmental conditions and may be rapid. Consequently, no assumptions regarding stability of state are permissible when tumor cell suspensions are manipulated *in vitro*, and it is imperative that each and every preparation and dilution thereof be subjected to microscopic examination at the time of inoculation when quantitative titrations for tumor-inducing activity are being carried out.

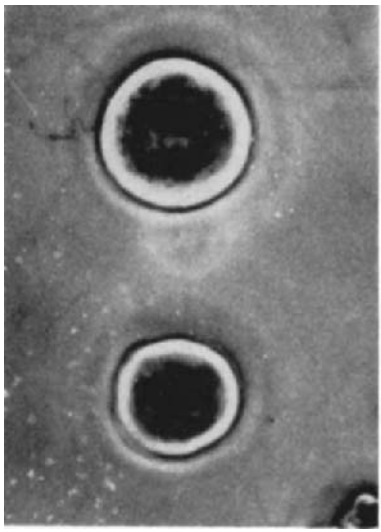

FIG. 1. Phase contrast, 8-mm. objective, N.A. 0.45.

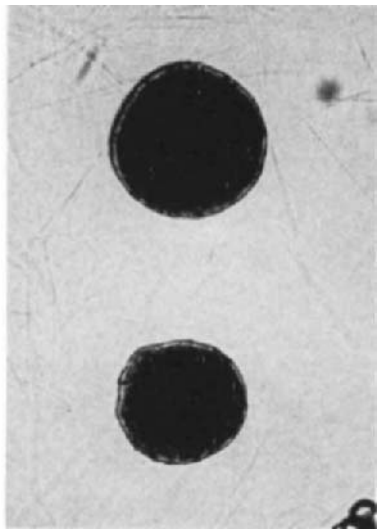

FIG. 2. 2536 Å, 6-mm. objective, N.A. 0.7.

FIGS. 1 and 2. S 37 cells in paramorphic state.

The distinguishing characteristics of α- and β-phase cells are indicated in Table II, and the appearances of the extreme α type under phase contrast illumination and at 2536 Å are shown in Figs. 1 and 2. It should be pointed out that the microscopy of α-phase cells is attended by certain optical and technical difficulties. Optical artifacts are accentuated if phase plates having too low a percentage transmission are used, and resolution is limited in hanging drop preparations. The technical difficulties are due to the rapid changes exhibited by α-phase cells in response to environmental changes which may be induced in preparing them for microscopic examination; for example, if peritoneal exudate is diluted with physiological salt solution and mounted between slide and cover slip, rapid oxygen depletion depresses the activity of α-phase cells and induces changes to the inactive paramorphic state.

A few simple factors determine phase transformation and change to

the inactive paramorphic state *in vitro*. These are summarized in Table III.

The influence of factors listed in Table III on cell state may be simply and clearly demonstrated with ascites tumor cells in a shallow hanging-drop preparation, provided that precautions are taken to prevent the suspending fluid from becoming hypertonic by evaporation and provided that a high standard of chemical cleanliness is observed in the washing

TABLE III

Factors Which Determine $\alpha \rightleftharpoons \beta$ Transformation and Transition to the Paramorphic State *in Vitro*

Change	Factors
α to β	Occurs at 20° to 37°C.; rate dependent on temperature. Supporting surface necessary, e.g., plasma clot, glass or quartz, other differentiated cells. Free access of O_2. Isotonic physiological salt solution with increased K:Na ratio.
α to P	Reduced temperature. O_2 deprivation. Hypertonic physiological salt solutions. Isotonic or hypertonic dextrose or glycerol solution.
P to α	Increased temperature. O_2. Replacement of dextrose solution with isotonic physiological salt solution.
P to β	Presumably P to α; then α to β.
β to α and β to P	Slow and infrequent in microscopic preparations. Cell usually dies under environmental changes employed to induce α to P transformation. Probably occur more frequently when solid tumor is cooled *en masse*.

of glassware and the preparation of the physiological salt solutions employed.

The available evidence indicates that only tumor cells in the paramorphic state are able to survive freezing to −79°C. (Craigie *et al.*, 1951; Craigie, 1952a,b). This conclusion is based on detailed quantitative studies with S 37, T 2146, C_3H sarcoma, and Bp 8 sarcoma (Craigie, unpublished) and is supported by less detailed observations on a number of other diphasic tumors—Daels guinea pig sarcoma, Jensen rat sarcoma, and a number of primary and transplanted C_3H mammary carcinomas. Table IV shows results obtained with a preparation of S 37 ascites tumor cells frozen for 8 days at −70°C. This particular example is selected for two reasons. One purpose is to show how rapidly tumors may develop from a few surviving tumor cells, provided that they are injected in a small volume with minimum of dead cells and tissue debris. The ultraviolet photomicrographs (Figs. 3 and 4) of the suspension used in this

experiment illustrate another point which will be discussed in Section IV.2.C. In this experiment S 37 peritoneal exudate was frozen in ampoules without additional diluent. After storage in the frozen state for 8 days it was quickly thawed at 37°C. without agitation. Rapid clotting occurred, and 9 minutes later the fluid exuding from the contracting clot was separated. A portion of this fluid was diluted with 22.5 parts of Ringer solution, and the diluted material was used immediately for ultraviolet

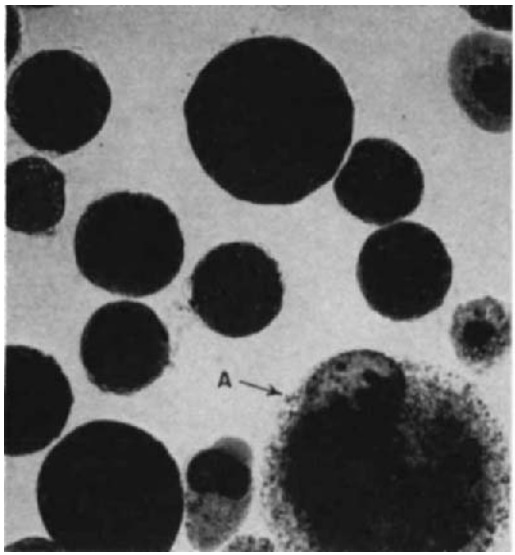

FIG. 3.

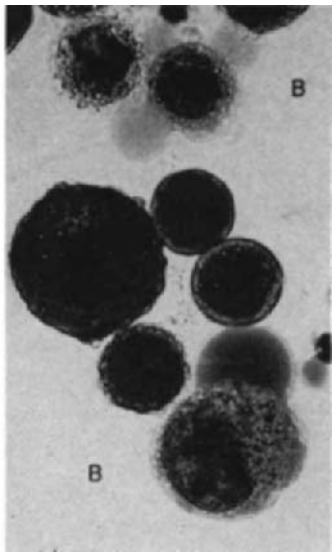

FIG. 4.

FIGS. 3 and 4. S 37 cells after freezing and thawing; 2536 Å, 6-mm. objective, N.A. 0.7; see Table IV and text. Fields selected to show: *A*, cell damaged by freezing; *B*, Loss of cytoplasmic absorption at 2536 Å and potocytosis.

photomicrography, cytometer count (under phase contrast), and mouse titration. Approximately 60% of the cells remained in the paramorphic state in the diluted preparation at the time of mouse inoculation. The results of mouse titration are shown in Table IV. Twenty C_3H mice were employed for the titration. Each mouse was inoculated at two subcutaneous sites with 0.01 and 0.001 ml. of the freshly thawed and diluted cell suspension. The remaining portion of the cell suspension which exuded from the clot was kept at 19°C. for 4 hours before being diluted and injected at two other subcutaneous sites in the same mice employed for titration of the freshly thawed preparation.

The observations of Craigie have been limited largely to a number of highly malignant tumors selected for investigation of the relationship be-

tween cell state and resistance to freezing because of the relatively high proportion of resistant or potentially resistant cells that they contain. In these diphasic tumors the main source of inactive paramorphic cells which are resistant to freezing are α-phase cells. In more differentiated tumors which are less resistant to freezing α-phase cells appear to be absent. Nevertheless some survival does occur, and this is associated with the presence of a few paramorphs in the frozen and thawed tumor suspension. In such tumors these resistant cells can be derived only from β-phase

TABLE IV

Volume Injected Subcutaneously*	Number of Cells Injected†	Mean Interval Till First Appearance of Tumors	Standard Deviation	Standard Error
0.01 ml. (16 sites)	5600	7.3 days	3.80	±0.95
0.001 ml. (14 sites)	500	9.5 days	2.43	±0.65
Undiluted ascitic fluid held at 19°C. for 4 hours and then diluted 1 in 23.5				
0.01 ml. (18 sites)	3200	7.5 days	2.40	±0.57
0.001 ml. (14 sites)	320	10.0 days	1.11	±0.30

* Micrometer syringe employed.

† Phase contrast count of cells in paramorphic state; figures may include some macrophages (probably less than 5%). Nonrefractile cells, which showed the dextrose effect on the nucleus and, in most instances, gross cytoplasmic changes, are not included in these counts.

cells, and it seems probable that susceptibility to freezing is due to the occurrence of lethal physical changes before any large number of these differentiated cells can adapt themselves to the changing environment. Investigation is difficult because of the small proportion of cells that do survive and the fact that a very small number (100 or less) may be capable of initiating a new tumor on transplantation of the treated tumor suspension.

3. Dehydration and Survival

In Sections II.2 and III.1 it was noted that Breedis suggested that his observations on the effect of rate of cooling on the survival of leukemic cells could be explained on the basis of partial dehydration. He remarked that "Initial freezing of water in extracellular fluids would result in the concentration of osmotically active material outside of the cell. Water would diffuse out of the cell to restore osmotic equilibrium leaving the protoplasm partially dehydrated. That such protoplasm may be a less favorable site for ice crystal formation has already been indicated." More

recent observations on the influence of glycerol media and rate of cooling on the survival of fowl and mammalian spermatozoa (Polge *et al.*, 1949; Polge, 1951; Smith and Polge, 1950; Polge and Rowson, 1952), red blood cells (Smith, 1950; Sloviter, 1951a,b; Mollison and Sloviter, 1951; Mollison *et al.*, 1952), rabbit ovarian granulosa cells (Smith, 1952), rat ovarian tissue (Parkes and Smith, 1953), and malpighian cells and epidermal melanoblasts of rabbit skin (Billingham and Medawar, 1951) support the view that some degree of cell dehydration is an essential factor in the development of resistance to freezing. However, as Parkes and Smith (1953) point out in their discussion of their observations on grafts of frozen ovarian tissue, other factors as yet not understood must play an important part in determining cell survival during freezing and at low temperatures. Hodapp and Menz (1951) investigated the respiration of liver and S 37 tumor slices exposed to liquid nitrogen. They found that liver cells are more readily killed under comparable conditions and that partial dehydration with ethylene glycol prior to rapid freezing quantitatively increases the survival of S 37 cells. Good correlation was observed between the morphological and respiratory characteristics of S 37 tumor cells after freezing, thawing, and incubation for 4 hours at 37°C. (Hodapp *et al.*, 1952). The respiration of tumor cells pretreated with ethylene glycol was 50 to 60% of the unfrozen controls, but when this pretreatment was omitted no significant respiration could be demonstrated after freezing.

More direct evidence that a considerable degree of cell dehydration is involved in the development of resistance to freezing is provided by observed changes in volume during α to P and P to α/β transformation. Craigie (unpublished) measured these changes in diameter of spherical cells (S 37 and C_3H/Bp 8) from interval photomicrographic records. He found that α cells decrease significantly in diameter in changing to the inactive P state and that when the environmental conditions are reversed to induce β transformation the original volume is regained before the cell loses its spherical shape. In the initial stages of transformation from the P state the changes in diameter indicate that cells may increase 50 to 100% in volume.

The observed differences in resistance of neoplastic and normal tissues to freezing and thawing do not necessarily indicate that there is any essential difference in the basic mechanism of survival. Certain neoplastic cells show a striking ability to adapt themselves to changing environmental conditions. When deprived of oxygen or exposed to hypertonic solutions they exhibit changes in volume and refractive index indicative of partial dehydration by spontaneous exosmosis. Normal cells lack this degree of adaptability and therefore are less resistant to freezing or succumb unless pretreated with hypertonic sugar or glycerol solutions.

4. Sequence of Changes during Freezing of Ascites Tumor Cells

The main points discussed in the preceding subsections are conveniently summarized by a description of the sequence of events that occur when an ascites tumor is frozen according to the method described in Section V. When peritoneal exudate containing the ascites tumor cells is placed in ampoules and these are sealed and cooled in a refrigerator at 4°C., oxygen depletion and the lowering of temperature cause the majority of tumor cells to shrink and assume the inactive paramorphic state. The decrease in volume of the cells is accompanied by a marked rise in refractive index, and these correlated changes indicate that a substantial loss of water occurs. The change to the paramorphic state is promoted by the addition of isotonic or slightly hypertonic dextrose solution (5.3 to 6%). It is desirable to chill the cell suspension before adding any large volume of dextrose solution. If the addition is made at room temperature, the dextrose solution should be added gradually, because too rapid changes of environmental fluid may kill active α- and β-phase cells. When the chilled cells have completed their adaptation at 4°C. (as shown by phase microscopy), the ampoules are transferred for freezing and storage to an insulated box containing CO_2 ice. It is not known whether a significant degree of further dehydration occurs during the period of freezing, but this is probable if a sufficient amount of extracellular electrolyte is present. During freezing the cells are exposed to the mechanical effects of ice crystal formation, as has been pointed out in Section III.1, cells in the paramorphic state are relatively resistant to the effects of pressure and shear. By the time the minimum temperature ($-70°$ to $-79°$C.) is reached, the extracellular fluid is frozen solid, and it seems unlikely that further extracellular physical changes can occur after this unless substances of low freezing point are present. On the other hand, the question of intracellular changes at this temperature remains a matter for speculation at present. It may be that the survival of a given cell depends upon the remaining water in that cell remaining unfrozen. This is possible if freezing is inhibited by high molecular weight substances, and in this connection the outstanding resistance of α cells is of interest, for these are characterized by the presence of a high concentration of PNA in the cytoplasm. Alternatively, cells may survive internal freezing if, as has been suggested earlier (Section III.1) vitrification takes place after supercooling to below $-55°$C.

IV. Preservation of Tumors in the Frozen State

1. Applications

The two main purposes for which low-temperature preservation of tumors may be employed are (1) long-term storage for maintenance of

tumor strains, and (2) storage of hemogenized tumor cell stocks for investigations in which standardization and quantitative control of tumor inoculum is desired.

1. In theory, the preservation of tumors in the frozen state would appear to be a preferable alternative to their maintenance by serial transplantation. Where it is necessary to maintain a number of tumor strains, a considerable saving in time, in animals, and in animal room space can be effected by the former method.

Unfortunately, reports on the survival of tumors stored in the frozen state for long periods are not numerous, but, on the other hand, they clearly indicate that tumors which are relatively resistant to freezing will survive for years in the frozen state. Ehrlich (1907) observed survival of a chondroma for 2 years. Breedis and Furth (1938) found a number of mouse tumors active after storage at −70°C. for periods up to 15 months. The cells of avian lymphoid tumors were found by Burmester (1950) to survive for long periods; in one instance tumors were obtained after a mean incubation period of 8.2 days in all of eleven chicks inoculated with material kept for 2028 days at −65° to −76°C. Burmester endeavored to determine whether there was a change in the growth activity of tumors after long-continued storage, and he had to conclude that any such changes were too small to be detected by the methods used. Craigie has experienced a similar difficulty. The longest periods of storage at −70°C. tested by Begg and Craigie (see Craigie, 1952b) are 43 months (S 37), 29 months (Daels guinea pig sarcoma), and 23 months (Walker rat tumor); in all instances tumor growth was obtained. Low-temperature storage has been extensively employed by Craigie *et al.* since 1949, and in no instance has any obvious deterioration been observed in tumor stocks stored in the frozen state. Determination of the rate of inactivation of tumor cells under the conditions of storage employed must therefore await the completion of long-term quantitative observations.

2. The method of preparing tumor cell suspensions in dextrose permits homogenized stocks to be distributed to ampoules and frozen. Such stocks are valuable for special experimental purposes because of the uniformity of activity and the feasibility of estimating the required dose accurately on the basis of a preliminary titration of dilutions of the thawed material in isotonic dextrose solution.

2. Methods

A. *Preparation of Tumor Suspensions for Freezing.* The methods to be outlined have been found suitable for all forty-one strains of transplantable tumors to which they have been applied. These tumors comprise sarcomas and carcinomas of mice and rats and a guinea pig sarcoma, but this series is not sufficiently comprehensive and it should be assumed that

the absence of failures merely reflects this fact. It is extremely probable that tumors will be encountered which cannot be preserved by present methods and that technical advances will depend largely on the investigation of such tumors. Some further comment on the principles involved in the preservation of tumors in the frozen state is therefore deemed to be more important than a detailed description of methods because these produce effects that should be checked by phase microscopy and not be taken for granted.

The aim of the methods to be outlined is to precondition the maximum number of tumor cells to freezing by inducing them to assume the inactive and resistant paramorphic state. It is possible to transmit tumors with frozen and thawed material that contains 100 surviving cells or less. Qualitative tests may therefore fail to reveal that survival has been minimal and that there has been no margin of reserve activity. The variations in resistance to freezing exhibited not only by different tumor types and strains but by individual tumors of the same strain, or parts of a single tumor, appear to be due to differences in tumor cell activity *in vivo* which determine adaptability to preconditioning.

In Section III.3 it was pointed out that cells may assume the inactive paramorphic state spontaneously under conditions of oxygen depletion. Change to this resistant state may also be brought about by chilling and exposure to dextrose solutions, and a combination of these preconditioning treatments greatly enhances the proportion of cells resistant to freezing. Two methods will be described, applicable to solid growths and fluid ascites tumors, respectively.

(a) *Preparation of suspensions from solid tumors.* Collect portions of tumor tissue, preferably from several animals, in a sterile container and chill for 15 to 30 minutes at 4°C. (approx.). Discard grossly necrotic portions, rinsing the fragments if necessary in 5.3% dextrose solution, but do not confine collection to "healthy" portions of tissue. When chilled, mince the tumor tissue in chilled 5.3% dextrose solution using scissors, extrusion through a coarse wire screen, or a pressure mincer loaded with a coarse and a medium grooved plunger (grooves 0.25 to 0.5 mm. deep). Add sufficient dextrose to give a 1 in 2 to 1 in 5 suspension; if a pressure mincer is used, place the required volume of dextrose solution above the plungers before mincing. Transfer the mince suspension to glass ampoules and seal. Place the ampoules at 4°C. (approx.) for ½ to 1 hour and then transfer to a CO_2 ice cabinet for slow freezing in cold CO_2 vapor. This method is suggested for tumors not previously tested for resistance to freezing or for tumors that do not survive well when reduced to single cell dispersions.

If experience has shown that frozen preparations of a given tumor normally retain an adequate reserve of activity, it may be preferable to use

a finer mince or free cell suspensions should the frozen stock be desired for purposes requiring the maximum uniformity of dosage.

(b) *Preparation of ascites tumors for freezing.* If peritoneal exudate is frozen without prior treatment, clotting may occur immediately after thawing with the result that the number of free tumor cells is greatly reduced. Rapid clotting also takes place when fresh peritoneal exudate is diluted with $1\frac{1}{2}$ to 4 volumes of dextrose solution but if the mixture is gently agitated while the precipitation of fibrin is in progress, the majority of cells escape entanglement and remain free. Exudate from a single mouse may be withdrawn into a 10- or 20-ml. syringe, followed by a half volume of dextrose solution and a small volume of air. The syringe is gently rocked until mixing is complete. Air is expelled, and a further small volume of dextrose solution is drawn into the syringe and mixed, this sequence being repeated until the desired dilution is obtained. The mixture is expelled into a tube which is kept in motion for a few minutes before distribution to ampoules.

The following method is used when frozen stock prepared from pooled ascites tumors is required. A glass pipette, drawn to a capillary point, is connected to a nitrogen supply adjusted to a slow rate of flow and inserted in a test tube of suitable capacity. The successive lots of peritoneal exudate are placed in the test tube together with an equal volume of dextrose, the mixture being kept in motion by the stream of nitrogen bubbles. When collection and dilution of the mixture is complete, the tube is stoppered, transferred to a low-speed shaker for 15 to 30 minutes, and then distributed to ampoules, chilled, and finally transferred to the CO_2 ice cabinet.

B. *Storage in the Frozen State.* There is a dearth of information about the optimum temperature of storage of tumors in the frozen state, particularly long-term storage. Klinke (1937) found that frozen tumors stored at $-20°C.$ lost their transmissibility in 3 weeks. Turner and Fleming (1939) found that spirochetes stored at $-78°C.$ retained their virulence after 3 years, although Turner (1938) found that at temperatures between $-20°$ and $0°C.$ inactivation was more rapid than at higher temperatures. Probably the observations of Hazel *et al.* (1949) on low-temperature studies with colloidal silicic acid (see Section III.1) are pertinent here; these suggest that tumors should be stored below $-55°C.$ Fortunately, CO_2 ice provides a convenient method of maintaining a sufficiently constant temperature below this level.

CO_2 ice refrigeration may with advantage be supplemented by electric cooling to reduce consumption of the expendable refrigerant. A brief description of a combined unit* which has been in operation in the author's laboratory since June, 1950, may be of interest. The storage cabinet is a

* The cost of this equipment was defrayed out of a grant from the Damon Runyon Memorial Fund for Cancer Research, Inc.

CO_2 ice box fitted with a horizontal stepped lid, operated by a vertical hoist. Insulation is provided by 12 inches of ozonote (expanded ebonite). The CO_2 ice is kept in three compartments, each measuring 8 × 18 × 18 inches, and tumor preparations are stored in specially designed racks arranged in two intervening compartments (18 × 18 × 18 inches). Each rack contains eleven shelves sloping downward toward the back of the rack at an angle of 20°. Interchangeable corrugated metal strips permit 3- or 5-ml. ampoules to be filed without risk of sideways displacement. Ampoules are identified by a color code, by means of colored plastic adhesive tape. A card index, which gives rack, row, place, and color coding, permits immediate location of any tumor stock. Within the storage space available there is accommodation for over 2700 ampoules. Electric refrigeration to −82°C. is powered by a 3 hp. motor and is controlled by a time switch. When this is set to operate for 2-hour cycles every 8 hours the consumption of CO_2 ice is 9 to 10 lb. per day.

C. *Thawing of Frozen Stocks.* Thawing must be carried out rapidly by immersing the ampoule at −70°C. (approx.) in water at 37°C. and keeping the contents in motion until thawing is almost complete so that the temperature does not rise initially beyond a few degrees above freezing point. Rapid changes may occur in some cells immediately after thawing; phase contrast refractility and cytoplasmic opacity at 2536 Å are lost, and degenerative changes follow (see Figs. 3 and 4). These changes occur in a greater percentage of surviving cells if large numbers have been damaged during preparation and freezing of the suspension. It is suggested that this may be related to the excess of K ions and compounds liberated from the intracellular fluid of the damaged cells.

V. Prospective Developments and Limitations

Existing limitations to the preservation of tumors in the frozen state are, in reality, the limitations of experience restricted to an insufficient range of transplantable tumors; but even improved methods of the future may prove to be inadequate for the preservation of all tumors. Other considerations suggest that under certain conditions freezing might promote tumor progression by exercising a selective effect. Although it remains for future investigation to show to what extent hypothetical possibilities may occur in practice, a brief discussion of these may be of some value to others interested in further development of methods of preserving tumors. The main purpose of this discussion is to indicate some of the lines along which future investigations might usefully proceed, but it may also serve as a caution against the empirical use of methods. It has been emphasized that some methods provide only minimal survival with tumors that are potentially highly resistant to freezing, and it should be obvious that the appli-

cation of such methods to other tumors is bound to yield unsatisfactory results.

It is not known whether the methods described in the preceding section for preparing tumors for preservation in the frozen state can be applied successfully to all tumors, or even whether the principles on which they are based are applicable. However, recent observations on normal tissues, particularly those on spermatozoa, red blood cells, and ovarian tissue (Section III.3), support the view that the solution of the problem of preserving a given kind of cell in the frozen state depends primarily on solution of the problem of preconditioning it prior to freezing. Successful preconditioning appears to depend on causing loss of some intracellular water without injury to the cell, in fluids which do not undergo alterations during freezing and thawing which are prejudicial to cell survival. Tumors which are relatively resistant to freezing also exhibit a considerable resistance to glycerol, but the possibilities here remain to be explored. The effects of sugar solutions other than dextrose, of glycols, and of the addition of amino acids or proteins as protective agents merit investigation. Gradual increase of tonicity during preconditioning may prove to be advantageous, and in this connection attention is drawn to the observations of Opie (1949a,b) on the high isotonicity of some tissues, e.g., liver (0.34 *M* NaCl) as compared with erythrocytes (0.15 *M*).

Tumors may change in the course of serial transplantations, acquiring characteristics of greater malignancy. Sarcomatous transformation of the stroma of mammary carcinomas of mice is not uncommon. Even in the primary host, changes may occur (Foulds, 1949). In the event of a few variant cells arising in tumors which have a significantly greater resistance to freezing than those from which they are derived, it is to be expected that progression will be favored. So far, there is no definite evidence that such a selective effect of freezing is likely to occur or prove a serious limitation to preservation of tumors in the frozen state. However, a recent report by Walsh *et al.* (1951) is suggestive and at least provides a warning against a somewhat unexpected risk involved in the treatment of tumors by freezing.

Walsh *et al.* (1950) in a study on the effect of low temperature on the morphology and transplantability of S 37, observed that, after several serial transplantations following an initial freezing treatment, this tumor failed to grow. These authors (1951) encountered this phenomenon on several occasions and state that two other investigators had similar results with frozen material supplied by them. Five failures, after several successful transplantations, were with tumor stored at −30°C. and three were with tumors stored at −70°C., the storage periods varying from 24 hours to 3 months. In contrast, Walsh *et al.* found that S 37 frozen in

liquid nitrogen for 15 minutes before storage at −70°C. could be carried through many transplant generations. These authors appear to conclude that fast freezing (approaching the several hundred degrees per second required to obtain an intracellular vitreous state, according to Luyet and Gehenio) is necessary to protect normal and neoplastic cells from the injurious effects of ice crystal formation. They consider a number of hypothetical possibilities in discussing their observations on growth failure, but examination of their technique suggests a much simpler explanation. Sarcoma 37 was prepared for freezing by cutting the tumor into small fragments and passing these through a syringe several times. "The needle size was gradually reduced until the mince would pass readily through a 24 gauge needle. No fluid was added and each animal received 0.1 *cc.* of the inoculum subcutaneously." Examination of the percentage of takes in the control group receiving unfrozen material indicates a most unsatisfactory initial activity. Percentage takes varied from 83 to 100%, and the average latent period was 10 to 13 days (*cf.* results with small number of S 37 cells, Table IV). With this unsatisfactory control base line it is impossible to judge what percentage of S 37 cells may have survived the freezing treatment employed by Walsh *et al.*, but many of the figures quoted in their communication appear to indicate minimal survival. Craigie (unpublished) has attempted unsuccessfully to reproduce this phenomenon which is probably due to the selective effect of minimal survival and not to damage or change in the tumor cells brought about by freezing. A possible explanation is suggested by the mitotic abnormalities that occur with great frequency in S 37 (Diller, 1952) and other degenerate transplantable tumors. It is probable that some of these, although not immediately incompatible with cell survival or subsequent mitoses, may lead eventually to death of the clones arising from cells in which they have occurred.

It remains for future investigations to show whether the risk of inducing tumor changes by freezing treatments is a significant one. In the long-term view, there would seem to be a greater risk in serial transplantation for 3 or 5 years than in storing a frozen tumor for the same period. The best safeguard would seem to be the employment of methods giving maximum cell survival, and to this end much further work is required. Existing methods, although adequate for many tumors, are obviously open to considerable improvement.

REFERENCES

Apolant, H. 1914. *In* Paul Ehrlich: Festschrift zum 60 Geburtstage, pp. 361–378. Gustav Fischer, Jena.

Auler, H. 1932. *Z. Krebsforsch.* **35,** 103–108.

Barnes, W. A., and Furth, J. 1937. *Am. J. Cancer* **30,** 75–94.

Begg, A. M., and Craigie, J. 1952; *see* Craigie, 1952b.
Billingham, R. E., and Medawar, P. B. 1951. Freezing and Drying, pp. 55–62. Symposium held June 1951, Institute of Biology.
Bittner, J. J., and Imagawa, D. 1950. *Cancer Research* **10,** 739–750.
Blumenthal, H. T., and Walsh, L. 1950. *Proc. Soc. Exptl. Biol. Med.* **73,** 62–67.
Breedis, C. 1942. *J. Exptl. Med.* **76,** 221–240.
Breedis, C., Barnes, W. A., and Furth, J. 1937. *Proc. Soc. Exptl. Biol. Med.* **36,** 220–224.
Breedis, C., and Furth, J. 1938. *Science* **88,** 531–532.
Briggs, R., and Jund, J. 1944. *Anat. Record* **89,** 75–85.
Burmester, B. R. 1950. *Cancer Research* **10,** 708–712.
Caspersson, T. O. 1950. Cell Growth and Cell Function. W. W. Norton & Co. New York.
Craigie, James. 1949a. *Brit. J. Cancer* **3,** 249–250.
Craigie, James. 1949b. *Brit. J. Cancer* **3,** 268–274.
Craigie, James. 1949c. *Brit. Med. J.* **2,** 1485–1491.
Craigie, James. 1950. *47th Ann. Rept. Imp. Cancer Research Fund* (1949–1950), pp. 5–18.
Craigie, James. 1952a. *J. Pathol. Bacteriol.* **64,** 251–252 (abstr.).
Craigie, James. 1952b. *49th Ann. Rept. Imp. Cancer Research Fund* (1951–1952), pp. 5–9.
Craigie, James. 1952c. *Ann. Roy. Coll. Surg. (Eng.)* **11,** 287–299.
Craigie, James, Lind, P. E., Hayward, M. E., and Begg, A. M. 1951. *J. Pathol. Bacteriol.* **63,** 177–178 (abstr.).
Cramer, W. 1930. *9th Sci. Rept. Imp. Cancer Research Fund*, pp. 21–32.
Des Ligneris, M. J. A. 1930. *Brit. J. Exptl. Pathol.* **11,** 249–251.
Diller, I. C. 1952. *Growth* **16,** 109–125.
Dmochowski, L., and Millard, A. 1950. *Brit. Med. J.* **2,** 1136–1137.
Ehrlich, P. 1907. *Z. Krebsforsch.* **5,** 59–80.
Foulds, L. 1949. *Brit. J. Cancer* **3,** 345–375.
Gabrielson, R. M., Syverton, J. T., and Kirschbaum, A. 1952. *Cancer Research* **12,** 117–123.
Gaylord, H. R. 1908. *J. Infectious Diseases* **5,** 443–448.
Gye, W. E. 1949. *Brit. Med. J.* **1,** 511–515.
Gye, W. E., Begg, A. M., Mann, I., and Craigie, James. 1949. *Brit. J. Cancer* **3,** 259–267.
Hazel, F., Parker, J. A., and Schipper, E. 1949. *Science* **110,** 161–162.
Hodapp, E. L., and Menz, L. J. 1951. *Anat. Record* **111,** 538 (abstr.).
Hodapp, E. L., Wade, J. N., and Menz, L. J. 1952. *Proc. Soc. Exptl. Biol. Med.* **81,** 468–475.
Klinke, J. 1937. *Z. Krebsforsch.* **46,** 436–455.
Klinke, J. 1939. *Growth* **3,** 169–172.
Klinke, J. 1940. *Klin. Wochschr.* **19,** 585–590.
Kreyberg, L., and Hansen, O. 1950. *Scand. J. Clin. & Lab. Invest.* **2,** 168–170.
Law, L. W. 1951. *Cancer Research* **11,** 795–800.
Ludwin, I. 1951. *Biodynamica* **7,** 53–55.
Luyet, B. J. 1951. Freezing and Drying, pp. 77–98. Symposium held June 1951, Institute of Biology.
Luyet, B. J., and Gehenio, P. M. 1938. *Biodynamica.* **1,** 1–92.
Luyet, B. J., and Gehenio, P. M. 1940. *Biodynamica* **3,** 33–99.

Luyet, B. J., and Hodapp, E. L. 1938. *Proc. Soc. Exptl. Biol. Med.* **39,** 433–434.
Luyet, B. J., and Thoennes, G. 1938. *Science* **88,** 284–285.
Mann, I., and Dunn, W. J. 1949. *Brit. Med. J.* **2,** 255–257.
Michaelis, L. 1905. *Med. Klin.* (*Munich*) **1,** 203–209.
Mider, G. B., and Morton, J. J. 1939. *Am. J. Cancer* **35,** 502–509.
Mollison, P. L., and Sloviter, H. A. 1951. *Lancet* **2,** 862–864.
Mollison, P. L., Sloviter, H. A., and Chaplin, H. 1952. *Lancet* **2,** 501–505.
Moran, T. 1926. *Proc. Roy. Soc.* (*London*) **A112,** 30–46.
Moran, T. 1929. *Proc. Roy. Soc.* (*London*) **B105,** 177–197.
Opie, E. L. 1949a. *J. Exptl. Med.* **89,** 185–208.
Opie, E. L. 1949b. *J. Exptl. Med.* **89,** 209–222.
Parkes, A. S., and Smith, A. U. 1953. *Proc. Roy. Soc.* (*London*) **B140,** 455–470.
Passey, R. D., and Dmochowski, L. 1950. *Brit. Med. J.* **2,** 1129–1134.
Passey, R. D., Dmochowski, L., Lasnitzki, I., and Millard, A. 1950. *Brit. Med. J.* **2,** 1134–1136.
Polge, C. 1951. *Nature* **167,** 949–950.
Polge, C., and Rowson, L. E. A. 1952. *Nature* **169,** 626–627.
Polge, C., Smith, A. U., and Parkes, A. S. 1949. *Nature* **164,** 666.
Salvin-Moore, J. E., and Barratt, J. O. W. 1908. *Lancet* **1,** 227.
Salvin-Moore, J. E., and Walker, C. E. 1908. *Lancet* **1,** 226–227.
Sloviter, H. A. 1951a. *Lancet* **1,** 823–824.
Sloviter, H. A. 1951b. *Lancet* **1,** 1350–1351.
Smith, A. U. 1950. *Lancet* **2,** 910–911.
Smith, A. U. 1952. *Exptl. Cell Research* **3,** 574–582.
Smith, A. U., and Polge, C. 1950. *Nature* **166,** 668–669.
Snell, G. D., and Cloudman, A. M. 1943. *Cancer Research* **3,** 396–400.
Strumia, M. M., and Hodge, C. C. 1945. *Ann. Surg.* **121,** 860–865.
Turner, T. B. 1938. *J. Exptl. Med.* **67,** 61–78.
Turner, T. B., and Fleming, W. L. 1939. *J. Exptl. Med.* **70,** 629–637.
Walsh, L. B., Greiff, D., and Blumenthal, H. T. 1950. *Cancer Research* **10,** 726–736.
Walsh, L. B., Greiff, D., and Blumenthal, H. T. 1951. *Cancer Research* **11,** 727–730.
Warner, P. T. J. C. P., Gostling, J. V. T., and Thackray, A. C., 1950. *Brit. J. Cancer* **4,** 396–404.
Webster, J. P. 1944. *Ann. Surg.* **120,** 431–449.

Energy and Nitrogen Metabolism in Cancer

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CONTENTS

I. Introduction

The profound effects which a malignant neoplasm often produces in its host have long been evident to clinicians and investigators. Some neoplasms distort normal structures and anatomical relationships. Others produce ulceration of epithelial surfaces that may cause exsanguinating hemorrhage or overwhelming sepsis. On rare occasions a neoplasm may damage an organ to such an extent that it is unable to function, and the host presents a symptom complex characteristic of ablation of that organ. Some neoplasms produce biologically active substances, identical with or similar to naturally occurring hormones that ultimately threaten the existence of the organism because of the profound pharmacological responses which they elicit.

Many malignant neoplasms and related diseases kill their hosts without producing any of these effects. Primary cancers in man are frequently destroyed either by surgery or various forms of irradiation but recur eventually because initial therapy was incomplete or ineffective in eradicating all viable neoplastic tissue. Such cases represent an increasingly important segment of the cancerous population. Although the

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clinical manifestations of the evolution of their disease vary, certain factors may be recognized which are common to all as the hosts progress toward death. Necropsy usually discloses metastases more or less widely disseminated throughout the body. Some of these metastases have produced death solely because of their anatomical locations. A large group remains, however, in which death must have been due to profound alterations in the metabolism of the host.

The nature of these alterations, with which are associated anorexia, cachexia, and the other symptoms and signs of terminal cancer, has been the subject of considerable investigative effort both in animals and in man. Since the body loses weight, body fat, water, and protein must be lost in most cases. These observations have focused the attention of some investigators on the nitrogen and energy exchanges in the tumor-bearing host. Much of the work concerned with these aspects of cancer has been poorly controlled. Even those studies which have been undertaken with the utmost care have yielded data concerning net changes alone and have not elucidated the intermediate steps. Nevertheless, the information which has been obtained has been useful in an understanding of the host-tumor relationship and in suggesting new avenues of approach to problems of neoplasia.

Nitrogen and energy metabolism are intimately associated in all organisms. There is evidence that any given class of foodstuff may supply portions of the molecules synthesized within the organism. Dynamic interrelationships among carbohydrate, fat, and protein are complex indeed. For the sake of simplicity, however, energy metabolism and nitrogen metabolism will be considered separately in this review and an attempt will be made to correlate them in the final discussion. The leukemias and lymphomas are included among the malignant neoplasms considered herein.

II. Energy Metabolism in Human Cancer

1. Clinical

Progressive growth of tissue requires energy. One characteristic of the advanced cancerous state is cachexia. The mechanisms by which this comes about are difficult to evaluate. Reduction of food intake as stressed by Willis (1948) is certainly important, but there is evidence that other factors operate as well. In 1869 Pettenkofer and Voit studied a leukemic patient whose energy production was high as measured by oxygen utilization and carbon dioxide excretion. They found that the nocturnal oxygen consumption represented 46% of the total oxygen consumed in 24 hours, and the CO_2 produced at night was 49% of the total CO_2 in

24 hours. This was a higher proportion than that found in healthy individuals. They concluded that a patient with leukemia was unable to reduce his oxygen demand as a normal person did but offered no adequate explanation for the phenomenon which they observed.

Wallersteiner (1914) published the first systematic study of energy metabolism in afebrile patients with advanced cancer. Twenty-six of the 33 patients described had carcinoma of the stomach. He used the Grafe apparatus to determine the exchange of carbon dioxide and oxygen under resting conditions for periods of 40 minutes to 10 hours. Five of the subjects showed considerable elevation of the metabolic rate and 15 of the patients produced more than the 30 cal./kg./day which Wallersteiner used as his standard of reference. Sixteen patients had energy expenditures between 26 and 30 cal./kg./day (within normal range) while only two patients had markedly reduced metabolic rates. These patients had the greatest body weights of all the patients studied and were well nourished individuals. Surgical removal of the carcinoma resulted in a reduction of the basal metabolic rate in one patient. Recurrence of the tumor was accompanied by an increased expenditure of energy.

Murphy, Means, and Aub (1917) summarized previous studies of energy metabolism in patients with leukemia. The basal metabolic rates reported were all elevated. Their patient had lymphatic leukemia. The basal metabolic rate was elevated when it was determined by both direct and indirect means. From their data they concluded that most of the calories were supplied by fat. This patient then received roentgen ray therapy and responded well clinically. The basal metabolic rate was reduced after treatment. Subsequent studies of patients with leukemia have confirmed these findings (Lennox and Means, 1923; Minot and Means, 1924). Gunderson (1921) found that the elevation of the basal metabolic rate could be correlated with the proportion of immature cells present in the blood stream.

Elevation of the basal metabolic rate in patients with other malignant neoplasms has been reported frequently (Boothby and Sandiford, 1922; Heindl and Trauner, 1927; Strieck and Mulholland, 1928). Heindl and Trauner found the metabolic rate to be highest in the patients with large tumors which had metastasized and were growing rapidly. In some of their cases the basal metabolic rate returned to normal levels following surgical removal of the primary cancer, rising later when the cancer recurred. In other patients the basal metabolic rate was normal. All these patients had operable cancers by their criteria of operability. Although there are many records of increased metabolic rates in patients with cancer at various stages of their disease, it is interesting that there are few reports in which the rates were significantly lowered. Most normal adults

ingesting 1500 cal. a day have a basal metabolic rate of approximately −15 to −20% (Benedict *et al.*, 1919; Keys *et al.*, 1950). Many cancerous subjects ingest far less than this at certain stages of their illness.

Waterhouse *et al.* (1951) studied eight patients with widespread malignant neoplasms of different anatomic sites. All the patients ingested diets which should have been adequate in all respects for normal individuals of the same age, sex, and body habitus engaged in similar activity. All except one patient, who was febrile, retained nitrogen. Caloric expenditures were calculated by the method of Newburgh (1942). Two of the patients were in negative caloric balance throughout the study. One patient with Hodgkin's disease was in negative caloric balance until she responded to nitrogen mustard therapy, when she began to expend fewer calories than she ingested. Another patient who had a net caloric deficit while eating 2700 cal. a day went into positive caloric balance when he ingested 3255 cal. daily. The four remaining patients were in positive caloric balance. Changes in body weight did not always reflect changes in body tissue. Some of the subjects retained large amounts of fluid while they were being studied. This work requires confirmation by some other means of determining caloric expenditure.

The presence of normal basal metabolic rates and the absence of weight loss in some patients with malignant neoplasms suggest that at certain stages of tumor growth the energy expenditure is not necessarily increased or that dietary intake keeps pace with any increase which occurs.

2. Experimental

Although a considerable literature exists on the effects of nutrition on the tumor-bearing host, much of this has concerned itself with specific dietary factors and relatively little with the problems of energy metabolism in animals. This is particularly true of studies of gaseous exchange, most of the information having been obtained by less direct means. Net changes during the life span of the tumor have been measured rather than changes which occur during various phases of progressive neoplastic growth.

Evidence has been presented that tumor transplants do not "take" or develop well in poorly nourished animals (Rous, 1914; Drummond, 1916, 1917; Voegtlin, 1937). However, if the dietary intake is reduced after the establishment of a transplantable tumor in its host or the development of a spontaneous tumor, the growth of the neoplasm may be little if at all affected by diets inadequate to maintain growth or equilibrium of the tissues of the host (Rous, 1914; Drummond, 1917). In some of this early work dietary restriction was not limited to restriction of available sources of energy but involved protein and vitamin deficiencies

as well. Drummond eliminated these objections by including water and alcohol and ether extracts of foods which contained essential factors for growth and adequate protein in his semisynthetic diets. His data unfortunately do not include the daily dietary intake and it is, therefore, impossible to determine whether the restriction of calories alone resulted in significant reductions in the incidence or rate of growth of the successful transplants.

Bischoff *et al.* (1935) demonstrated that a 50% restriction of dietary intake resulted in considerable inhibition in growth of transplanted sarcoma 180 in mice fed a commercial calf meal. They do not state whether restriction began before or after inoculation. They attributed their results to restriction of calories but objection was raised to such an interpretation on the basis that total food restriction had occurred, not simple caloric restriction. In subsequent studies (1938) Bischoff and Long fed the same basic diet to two groups of mice bearing sarcoma 180. In one group they supplemented this with Crisco or cornstarch to increase the caloric intake. The mice on the restricted caloric intake lived longer and the sarcomas grew more slowly. A third group of mice was fed cornstarch alone for 14 days. This resulted in marked weight loss and retardation of tumor growth. Tannenbaum (1942) studied the effect of caloric restriction on the development and growth of a spontaneous breast carcinoma and chemically induced tumors in mice. The basic diet was adequate to maintain body weight but not to support growth in the sense of weight increment. Additional calories were supplied to one group of animals in the form of cornstarch. The restricted group had a much lower incidence of both spontaneous and induced tumors, the tumors grew more slowly when they appeared, and longevity was increased. These studies utilized groups rather than individual mice. Results were expressed as average values of the groups. The differences between them were statistically significant.

Studies of energy and nitrogen metabolism in individual rats bearing transplanted Walker carcinoma 256 revealed that food intake decreased with progressive tumor growth, carcass weight was lost progressively, and there was a loss of body fat (Mider *et al.*, 1948). The loss of body fat during the period of tumor growth was much greater than that of their controls which were of the same age and sex, weighed the same amount at the time of tumor implantation, and ingested identical quantities of the same diet (Mider *et al.*, 1949). This suggested that the presence of a progressively growing neoplasm increased the caloric expenditure of the host. The caloric value of the amount of fat lost from the body during tumor growth was later shown to be equal to the total calories lost from the rat's normal tissues, during tumor growth as determined by bomb

calorimetry of the rat carcass and chemical determination of total carcass lipids (Mider *et al.*, 1951). It seems clear from analyses of carcasses of rats bearing Walker carcinoma 256 killed at various stages of tumor growth that the progressive loss of lipid begins only when anorexia has developed (Mider, 1953).

Haven *et al.* (1949) stated that the proportion of total carcass lipid varied inversely with the size of the tumors in rats bearing Walker carcinoma 256 and that the concentration of blood lipids was often markedly increased, the principal increase being in the saponifiable fraction. They showed subsequently (1951) that the lipemia reached a peak during the course of neoplastic growth and then declined to normal values just before the death of the subject. Begg and Dickinson (1951) also noted lipemia in rats bearing Walker carcinoma 256 which was not present in noncancerous animals fed the same amounts of the same diet. Adams (1950) found that CBA mice bearing the Gardner lymphosarcoma developed fatty livers if they were fasted for 48 hours. This occurred only during the middle period of the growth of the tumor and was absent in the early and terminal stages.

It may be that the lipemia reflects an increased mobilization of fat to meet the increased demands for energy and disappears when the fat stores are essentially exhausted. This may also explain why the lipid content of the Murphy-Sturm lymphoma, the Walker carcinoma (Mider *et al.*, 1948) and the Gardner lymphosarcoma (Adams and White, 1950) decreases as the tumor becomes larger. Reduction in body lipid stores may reduce the available lipids so that the tumor is able to store less in proportion to its rate of growth. It is interesting that when a high fat diet was fed (72% of total calories) to rats with Walker carcinoma the tumor maintained a constant lipid concentration (Haven *et al.*, 1951).

Attempts have been made to maintain a large caloric intake by gavage using a high fat diet (Ingle *et al.*, 1947) to determine whether the changes which are observed in the natural course of tumor growth can be altered (Begg and Dickinson, 1951). In Begg's series the tumors reached 20–24% of the body weight and the noncancerous tissues gained as much weight as did the normal animals. The adrenals, however, increased in size, the rats developed anemia and hepatic catalase activity was diminished. These findings corresponded to those in tumor-bearing rats which are not force fed, and the changes were not present in the controls. Mider (1951) fed Ingle's high fat diet substituting lactalbumin for egg albumin to circumvent the possibility that a high intake of avidin might impede tumor growth. The daily intake was approximately 50 cal. as compared to 70 cal. in Begg's series. Most of the animals developed anorexia, but two rats maintained their appetites until the tumors had reached 20%

of the body weight. The carcasses of these two rats weighed less than those of their controls which had ingested an identical amount of the same diet. The difference between the findings of Begg and of Mider is doubtless due to the difference in caloric intake.

Stewart (1952), working in Begg's laboratory, extended these observations by force-feeding high fat, high carbohydrate, or high protein diets developed by Ingle *et al.* (1947) to groups of rats weighing approximately 200 g. at the beginning of tumor growth. Each animal received 70 cal. a day. The experiments were terminated when the neoplasms had attained a weight approximately 25% of the whole (rat + tumor). The carcasses weighed slightly less than did the bodies of the noncancerous control rats. Carcass weight loss was not prevented. Stewart thought that the caloric intake was more important in determining the effects of diet on weight changes in the cancerous host than was the composition of the ration as long as the dietary ingredients were qualitatively adequate for growth of normal rats. One striking difference in the reaction of the rats to the various regimens was observed. Stewart found, as had Begg and Dickinson, that rats bearing the Walker carcinoma could not tolerate the indefinite administration of the high fat diet at a level of 70 cal. a day. Normal rats handled this ration without apparent difficulty but the tumor bearing animals developed profound metabolic disturbances which included an extreme hyperlipemia when the tumor weight approached 25% of the whole rat.

3. Summary

It is evident in some cancer patients and animals with some tumors that the expenditure of energy is increased at certain periods in the progressive growth of malignant neoplasms. It is possible that the basal metabolic rate in cancerous man does not fall with a reduced dietary intake as it does in normal human beings on a restricted caloric diet. Fat depletion occurs and lipemia develops during the course of tumor growth. These changes represent profound metabolic alterations.

III. Nitrogen Metabolism in Clinical Cancer

1. Balance Studies

In 1889 Müller determined the total nitrogen excretion of four women who ate no food or so little that he considered their dietary intakes negligible. Three of them had psychoses and the fourth had an esophageal stricture following the ingestion of lye. He compared the range of values obtained per unit of body weight with data obtained from the study of seven women and one man with advanced cancer, all but one of whom

succumbed to the illness shortly after the studies were completed. Two of the patients, one with carcinoma of the head of the pancreas and the other with disseminated cancer of the breast, excreted no more nitrogen than did the four women without neoplasms. Each of the other patients, however, excreted considerably more nitrogen than did the reference group. In one of these, a man with carcinoma of the penis, a diet containing 3067 cal. and 20.78 g. of nitrogen was inadequate to establish nitrogen retention. All these patients were extremely complex from a clinical point of view, and all but one had reached a terminal stage in his illness. With the exception of the patient just mentioned, they were ingesting low caloric diets.

Wallersteiner (1914) studied the nitrogen balances of 12 patients with advanced cancers. Although the periods of observation were short and abrupt changes in dietary intake occurred in some of his patients, his observations are of considerable interest. Seven of the 12 patients attained nitrogen equilibrium or positive nitrogen balance during the studies. Wallersteiner concluded that loss of nitrogen occurred only when the dietary intake was inadequate to meet the demands of the subject and stressed the importance of the relationship of nitrogen metabolism to energy expenditure, recognizing that the increase in total metabolism which he observed increased the energy requirements as well as the needs of the body for nitrogenous substances. He also suggested that the growth of the neoplasm was responsible for the retention of nitrogen by some of his subjects.

Moraczewski (1898) studied a patient with chronic myelogenous leukemia, determined the dietary intake of nitrogen, chloride (as sodium chloride), phosphorus, and calcium and measured the excretion of these substances. He found that large quantities of nitrogen and phosphorus were retained as the leukemia progressed. Milroy and Malcolm (1898) and White and Hopkins (1899) observed that the retention of phosphorus in patients with leukemia was greater in proportion to the amount of nitrogen retained than it was in normal individuals fed a similar diet. Subsequent investigators found that the retention of nitrogen and phosphorus was greater during the more active phases of leukemia and that the excretion of these substances increased appreciably in patients who had remissions of their disease following successful therapy with radium or with roentgen rays (Henderson and Edwards, 1903; Königer, 1906; Knudson and Erdos, 1917). Ordway (1919) observed the same phenomenon in patients with Hodgkin's disease. The amount of phosphorus excreted was proportionally greater than the amount of nitrogen. Analysis of blood from leukemic patients revealed a high phosphorus content, the level being higher when the proportion of immature cells was increased

(Buckman *et al.*, 1925). The increased retention of nitrogen and phosphorus during rapid growth of leukemic cells and the increased excretion of these elements during effective therapy were thought to be related to the composition of the leukemic cells, but in none of these earlier studies were the data sufficient to permit even a first approximation of quantitative changes or to determine what role, if any, the host played in the growth of the neoplasms.

Recent investigations have been based on principles set forth by Reifenstein *et al.* (1945). An individual fed a constant diet will achieve a dynamic equilibrium among the various body compartments. Cells contain nitrogen, phosphorus, and potassium in amounts which are characteristic of the particular tissues of which they are components. Storage or loss of protein is associated with retention or excretion of phosphorus and potassium in the proportion in which the three elements exist in the principal tissue or tissues which are being built or destroyed. Since phosphorus is intimately associated with active metabolism of bone, a correction must be made for the phosphorus which is bound to calcium in bone. Skeletal muscle makes up the bulk of the protein mass of the body; therefore, the ratio among nitrogen, phosphorus, and potassium found in muscle may be used for all practical purposes to indicate changes in the mass of protein in the individual. Albright and other investigators have shown the essential validity of this thesis since these elements are stored or excreted in such proportions by normal individuals and those with certain non-neoplastic diseases (Reifenstein *et al.*, 1945; Albright *et al.*, 1946, 1949, 1950; Waterhouse *et al.*, 1949; Eckhardt and Davidson, 1950). Calculations must be made on a balance basis; that is, the algebraic difference between ingestion and excretion of a given substance. It is of the utmost importance that adequate time be allowed for equilibrium to be reestablished when any change is made in the regimen else results cannot be correctly interpreted.

Studies by Pearson *et al.* (1949, 1950, 1951), Waterhouse *et al.* (1951), Adams *et al.* (1952), Fenninger *et al.* (1953) have demonstrated that nitrogen is readily stored by patients with progressively growing malignant neoplasms. Retention of phosphorus and potassium during tumor growth was excessive in relation to the amount of nitrogen retained. Regression of neoplasms following effective therapy was accompanied by excessive excretion of phosphorus and potassium. These findings suggest that the ratio of phosphorus and potassium to nitrogen is higher in tumors than in normal tissues, particularly muscle. This has been observed in the tumors which have been analyzed (Pearson *et al.*, 1949; Eliel *et al.*, 1950; Fenninger and Waterhouse, 1951; Fenninger *et al.*, 1953; Waterhouse *et al.*, unpublished data).

Such differences together with metabolic balance data have been used by Pearson and Eliel (1951) to determine sources of nitrogen lost during therapy, and by Fenninger *et al.* (1953) to determine the partition of nitrogen between the host and the neoplasm. They have found that all the nitrogen retained from the diet and additional nitrogen obtained from normal tissue were used for tumor formation in a patient with leukemia and one with lymphosarcoma. When therapy was effective and the tumors regressed, the excretion of nitrogen, phosphorus and potassium rose. The ratios of these elements, when ACTH or cortisone was used to bring about a remission in leukemia and lymphosarcoma, were similar to those found in the neoplastic tissue early in the course of therapy. With continued therapy, however, the ratios lay between those of muscle and the tumor tissues, approaching those of muscle as the tumor mass became smaller.

In one patient with lymphosarcoma to whom a nitrogen mustard was given, the loss of nitrogen, phosphorus, and potassium was found by calculation to be derived entirely from tumor tissue. The amount of nitrogen calculated as being stored by normal tissue during this period of tumor regression exceeded that retained from the diet (that is, the positive nitrogen balance was less than the total amount of nitrogen which was calculated as being utilized in the formation of the tissues of the host). This suggests that during the regression of a tumor some of the building blocks liberated from the neoplasm may be incorporated in the host's tissues. Direct proof of this is lacking at the present time, but it is a point of considerable significance and requires further investigation.

If the growing neoplasm is able to obtain building blocks from the tissues of the host when the total ingested food becomes inadequate to meet the demands of both, one might anticipate that this would be reflected in changes in the protein stores of the host. Changes have been observed in the more labile portions of the plasma proteins, among the principal body proteins for which we possess means of fairly accurate measurement.

Diminished antibody titers in Hodgkin's disease were reported by Wallhauser (1933). Dubin (1947) discussed the poverty of the immunological response of patients with Hodgkin's disease to lues, tuberculosis, typhoid, and brucella antigens and their increased susceptibility to infection. Forkner (1938) mentioned decreased antibody titers in patients with leukemia. Parfentjev *et al.* (1951) found that sera from 80% of people with non-neoplastic diseases agglutinated with Proteus antigen, whereas the sera from only 28% of the cancer patients studied did so. Balch (1950) however found that 16 of 18 patients with carcinoma of various sites had an anamnestic reaction to a standard dose of diphtheria

toxoid which was equal to that of 19 well nourished subjects. The patients with cancer were all poorly nourished individuals, and had low serum albumin concentrations. There appeared to be no relationship between initial antibody levels, serum protein concentration, albumin or globulin levels and the magnitude of the antibody response. Antibodies to diphtheria toxoid were produced in the presence of negative nitrogen balance. He was unable to demonstrate a relationship between the capacity to produce antibodies to toxoid and the development of other infections.

A hierarchy exists among the uses of protein in the animal economy. The priorities appear to be altered in response to poorly defined stresses. The work of Whipple and his co-workers (Madden and Whipple, 1940; Whipple, 1942) and of Cannon (1950) has shown that reduction of dietary intake to the point of protein depletion, depletion by plasmapheresis, or the elimination of essential amino acids from the diet produce profound effects on production of plasma proteins, antibodies, and resistance to infection. Most patients with cancer suffer from inadequate alimentation during the course of their illness. This must be taken into account in evaluating immunological responses and the other changes which occur in the plasma proteins and erythrocytes of patients with cancer. The mechanism of these changes, however, is probably not fully explained by diminished dietary intake, and certain factors remain obscure.

2. Hypoalbuminemia

Studies of the plasma proteins in patients with clinical cancer or related diseases suggest that protein depletion has occurred. Numerous reports of the concentration of plasma proteins in cancerous people are available and were reviewed by Huggins (1949).

The clinical procedures used to obtain the data for many of these reports have inherent defects which seriously interfere with accurate determination of albumin in pathological sera. The method of electrophoresis appears at the present time to be the single procedure which permits the simultaneous measurement of several protein variables in the plasma with highly reproducible results. Therefore, only those data obtained by this method will be considered in this review (Seibert *et al.*, 1947; Petermann and Hogness, 1948; Petermann *et al.*, 1948; Dillard *et al.*, 1949; Mider *et al.*, 1950). The remarkable changes which characterize the plasma proteins of patients with some plasma cell myelomas (Adams *et al.*, 1949; Dent and Rose, 1949) are rarely found in other neoplastic diseases and are therefore beyond the scope of this discussion.

Hypoproteinemia of moderate or marked degree is usually found in patients with advanced cancer. Smaller decreases in total protein concentrations may be present in earlier stages (Huggins, 1949). It is gen-

erally agreed that most cancer patients have a significant degree of hypoalbuminemia even when the neoplasm is localized. The decrease in plasma albumin proceeds more rapidly than the diminution of the total proteins, indicating a concomitant rise in total globulins. Fibrinogen generally increases more than the other globulin fractions. The concentrations of the alpha globulins vary directly with the total globulin concentration while the beta and gamma globulins lag behind the increase in total globulins (Mider *et al.*, 1950). Late cancer is sometimes associated with levels of gamma globulin which are lower than those found in healthy adults (Seibert *et al.*, 1947; Mider *et al.*, 1950). The alterations which occur in the plasma proteins appear to be constant among different types of malignant neoplasms, with some exceptions. Higher concentrations of gamma globulins, for example, accompany Hodgkin's disease more frequently than the other neoplastic states which have been extensively studied (Petermann *et al.*, 1948).

Although it is possible that the low serum albumin concentration might be related to a defect in the albumin in cancer patients, no evidence has been presented that albumin in the serum of these patients differs from that of normal subjects. Petermann and Hogness (1948) reported a plasma component in patients with gastric and pulmonary cancers which migrated rapidly when analyzed by electrophoresis in acetate-chloride buffer at pH 4. This substance is probably related to the plasma mucoproteins described by Winzler and Smyth (1948) which appear in high concentration in various pathological conditions but particularly in cancer and infections with pyogenic bacteria (Winzler and Smyth, 1948; Mehl and Golden, 1950).

Changes in plasma protein concentration, although characteristic, are by no means pathognomonic of a malignant neoplastic state. Similar alterations occur in other chronic, wasting diseases (Seibert *et al.*, 1947). This does not mean that the mechanisms responsible for hypoalbuminemia need be identical in all types of cancerous subjects nor in cancer and other wasting diseases.

3. Mechanism of Hypoalbuminemia

The mechanism whereby hypoalbuminemia is produced in malignant neoplasms is unknown. It does not appear to be related to faulty intestinal absorption, for the fecal nitrogen in patients with certain cancers or related diseases is not increased (Waterhouse *et al.*, 1951) even among patients with ulcerating lesions of the upper gastrointestinal tract (Ariel *et al.*, 1943; Ariel, 1949). Simple starvation in the sense of caloric and protein restriction is not necessarily associated with hypoalbuminemia (Taylor *et al.*, 1949; Keys *et al.*, 1950). Both decreased formation

and increased utilization or destruction may be implicated in the production of hypoalbuminemia.

Since the work of Whipple and others has indicated that the liver is probably the seat of production of serum albumin, many studies of liver function in cancerous subjects have been undertaken (Abels *et al.*, 1942; Ariel and Shahon, 1950; Tagnon and Trunnell, 1948; Popper and Schaffner, 1950). Some degree of impairment of hepatic function, as measured by one or more of rather standard tests, was found in a high percentage of cancer patients. These reports all suffer from the same basic problem, namely, a suitable definition of hepatic dysfunction and adequate means of measuring it. Most of the tests employed by these investigators have been shown to be related to changes other than those in the liver. It is possible to have persistent abnormalities in certain tests following apparently complete recovery from acute hepatitis for periods as long as 27 years (Klatskin and Rappaport, 1947). Good health and full activity were maintained by these patients for long periods of time.

Abnormalities in certain liver function tests are found in many diseases in which the liver is not the primary seat of pathological change. It is an organ which seems to be primarily concerned with maintaining a substrate that can be used by the cells of the organism. Molecules are broken down and rearranged into others better suited to the body's needs. Any diseased organism is subjected to stresses that seldom operate during health, and it appears likely that the liver as well as other organs and tissues adapts its activities to the new environment or is modified by the noxious stimulus. Abnormal liver function tests in cancer may reflect changes in the relative importance of metabolic processes in the cancerous host rather than pathological changes in the liver cells themselves. It is quite possible that hypoalbuminemia is the result not of loss of capacity of the liver to synthesize albumin but of the diversion of the nitrogenous building blocks to the formation of other proteins or to energy-producing substances in the cancerous individual. Although no final statement can be made, the evidence available does not indicate unequivocally that hypoalbuminemia is due principally to the inability of the liver to fabricate albumin.

Few data are available which throw any light on the problem of increased utilization of albumin itself by conversion to other proteins or deamination and eventual "burning" as a major factor in the development of hypoalbuminemia among cancerous individuals. Adequate study of this problem requires the intravenous administration of albumin as the principal if not the only source of protein and measurement of its utilization by the body. Plasma proteins given parenterally as the sole source of nitrogen can maintain dogs in good health for at least three months when

caloric requirements of the dog are met (Terry *et al.*, 1948). Parenteral administration of plasma proteins to hypoproteinemic human beings for shorter periods of time resulted in the retention of nitrogen. Phosphorus was also retained in an amount equivalent to that which would be retained if 50% of the injected plasma protein had been converted to tissue protein (Albright *et al.*, 1946).

Oral administration of human serum albumin to normal human subjects in amounts of approximately 1 g./kg. of body weight daily for 10 days as the only dietary source of protein resulted in nitrogen retention when a total of 3000 to 3300 cal. daily was supplied for energy metabolism. However, when the albumin was reduced to slightly less than 0.5 g./kg. of body weight per day, nitrogen was lost. Highly purified human albumin contains a minute quantity of tryptophan and little isoleucine. Supplementation of the albumin fed to the human subjects with essential amino acids failed to alter its utilization appreciably. Albumin retained in the plasma disappeared at a rate of approximately 50% in four to six days (Eckhardt *et al.*, 1948). Subsequent studies by Waterhouse (1949), Eckhardt and Davidson (1950) have in general confirmed these observations.

It would appear that a certain portion of the albumin is excreted in the urine in the absence of evidence of permanent renal damage (Terry *et al.*, 1948; Waterhouse *et al.*, 1949; Gimbel *et al.*, 1950). Large amounts accumulate in the plasma, interstitial fluid, and lymph which gradually disappear after the cessation of parenteral administration. A portion may be incorporated in body proteins without being degraded as suggested by the work of Whipple in dogs and Eckhardt and Davidson (1950) in man when the amounts of albumin are small or moderate. Another portion is degraded to its component amino acids which are either resynthesized to tissue protein or deaminated, the nitrogen excreted, and the carbon residues "burned" for energy. The relative amounts which suffer these various fates depend apparently on the needs of the individual, a depleted one retaining more of the administered albumin to synthesize tissue protein than a well-nourished subject. The 50% disappearance time of 4 to 6 days found in normal subjects was also found in two malnourished subjects, one with hepatic cirrhosis and one with carcinoma of the larynx (Eckhardt and Davidson, 1950). Waterhouse studied the disappearance of albumin in a patient with advanced metastatic mammary carcinoma and found that the 50% disappearance time lay within the 4- to 6-day range (personal communication). Further investigation may disclose some cancerous patients who "burn" and "convert" albumin more rapidly. Abnormally rapid "burning" of albumin has been observed in cases of "idiopathic" hypoproteinemia (Albright *et al.*, 1950) and in one

of Waterhouse's cases (1949) with generalized polyserositis who was febrile during the course of study. Excessive protein catabolism was found by a different technic in a patient with "idiopathic" hypoproteinemia (Kinsell *et al.*, 1950).

Hypoalbuminemia is by no means limited to cancerous individuals. It accompanies most acute infections. It is an early consequence of the reaction to injury (Peters, 1946). Trauma may cause a prompt and marked disturbance in the concentration of plasma proteins and in their relative proportions (Cuthbertson and Tompsett, 1935). Acute depletion by plasmapheresis and chronic depletion by low protein diets produce similar changes in the plasma protein pattern of dogs as studied by electrophoresis. Albumin production lagged behind the synthesis of other plasma proteins during repletion (Zeldis and Alling, 1945; Zeldis *et al.*, 1945). Changes in the body's protein metabolism which produce similar changes in the plasma protein picture, then, can be brought about by a variety of disease entities. The studies, however, have all measured overall effects, and we possess no knowledge of the intermediate events. An additional factor which might alter the concentration of the plasma proteins in patients with cancer could conceivably be changes in plasma volume (Mider *et al.*, 1950). Unfortunately no data are available which consider the effects of malignant neoplasms on the total circulating plasma proteins rather than on concentration. Much more investigation is needed to elucidate the many unknown factors in the mechanism of hypoalbuminemia among cancerous subjects.

4. Anemia

The relationship of the anemia found in approximately 75% of all patients at some time during their course of clinical cancer (Sturgis, 1948) and nitrogen metabolism is not clear. In certain patients the anemia is related to chronic blood loss, being hypochromic and microcytic in character. Carcinoma of the stomach may be associated with macrocytic and normocytic anemias which were attributed by Oppenheim *et al.* (1945) to hepatic dysfunction. Some cases of true pernicious anemia have been associated with gastric cancer. This relationship has been discussed by Barrett (1946).

Extensive involvement of bone marrow by metastatic carcinoma may be accompanied by severe anemia (Commons and Strauss, 1948). The usual explanation of this finding, the replacement of marrow cells by tumor masses, may be too facile. If this were the principal mechanism one would expect a reduction in other formed elements of the blood as well as erythrocytes and development of extramedullary hematopoietic foci as in those diseases where there is unquestionable encroachment on the

marrow by bone. Neither of these phenomena occurs commonly even when extensive bony metastases are readily demonstrable. Shen and Homburger (1951) concluded from their studies of 193 patients with advanced cancer, 60% of whom had reduced hemoglobin concentrations, that 28.5% of the anemias were due to blood loss alone, 56% were of the myelophthisic type, 2.6% due primarily to hemolysis, and 12.9% had characteristics of both blood loss and disturbances of function of the marrow. Osseous metastases were present in 50 patients, 24 of whom had anemia. The authors concluded that anemia associated with cancer is not usually the result of replacement of the bone marrow by neoplastic cells.

It has been commonly stated that the anemia associated with leukemia is myelophthisic. Jaffe (1933) found the erythropoietic activity in myeloid leukemias so great that it approached the leukopoietic activity in intensity in some cases and even surpassed it in acute myeloid leukemia. He concluded that hemolysis was the principal cause of anemia in leukemia, interpreting intense hemosiderosis he observed as evidence of excessive destruction of erythrocytes in spite of hemorrhagic diathesis prior to death. He subsequently reported cases in which a profound anemia was present in patients with acute leukemia which was out of all proportion to the involvement of the bone marrow by leukemic cells (Jaffe, 1935) and stressed again hemolysis and erythrophagocytosis as major factors in the pathogenesis of the anemia. He also pointed out that the anemia may antedate the development of extensive leukemic infiltrations in leukemia. Collins and Rose (1948) believed that increased but abnormal erythropoiesis accounted for the anemia observed in acute and chronic myeloid leukemia. They ascribed the anemia of acute lymphatic leukemia to extensive lymphocytic infiltration of the marrow as well as abnormalities of erythropoiesis. Blood loss or excessive destruction of erythrocytes aggravated the preexisting condition. Ross (1951) has found that the life span of the erythrocyte is markedly reduced in leukemia and malignant lymphomas even when there is evidence of little or no hemolysis. He suggests that the formation of the red cells is defective and that the anemia results from this defect even with a hyperplastic marrow. It is conceivable that the defect in the erythrocytes is due to a deficiency of building blocks for the red cell because of the severe competition offered by the leukemic cells for a limited substrate. Hence, the anemia may be intimately related to the other disturbances which occur in protein metabolism during progressive neoplastic growth. Further evidence for this is not yet available. It is probable that the anemias associated with malignant neoplasms will be found to have several causes when more is known about the factors involved in red cell formation and destruction.

IV. Nitrogen Metabolism in Experimental Cancer

The results of early studies of nitrogen metabolism in cancerous animals generally agreed with those in man. Moreschi (1909) found that the bodies of tumor-bearing animals lost weight when the animals were observed from the time that the tumors were implanted until death ensued. He felt that the tumor depleted the host but was unable to determine how this came about. Cramer and Pringle (1910) studied nitrogen balances in rats with transplanted Jensen sarcoma during the first two weeks of tumor growth. They found that nitrogen was retained in greater quantity during tumor growth than it was in normal animals eating the same food. Their meager data suggest rather than prove this thesis. No evidence was obtained from their studies that the tumors grew at the expense of the host nor was there a greater affinity of the tumors for building materials. They suggested that the nitrogen for the tumor was made available because of a "sparing action" on protein metabolism. This refers to a greater increment of mass per unit of nitrogen retained in the tumor-bearing rat and probably reflects the higher water content and lower nitrogen concentration of the tumor they studied. They recognized that effects during the later course of tumor growth were quite different but did not study them.

Several observers have confirmed Moreschi's findings and have reported that the body weight (rat minus tumor) declined as the tumors grew, suggesting that the tumor grew at the expense of the host. The specific constituents that were lost from the body, however, were not defined (Medigreceanu, 1910; Mischtschenko and Fomenko, 1928; McEuen and Thompson, 1933; Ball and Samuels, 1938). White (1945) demonstrated conclusively that a transplantable mouse mammary tumor could obtain enough nitrogen for its growth when the diet contained almost no nitrogenous substances and the animals were in negative nitrogen balance. The nitrogen for the formation of tumor protein must have come from the tissues of the host. The Walker rat carcinoma 256 also grew, although at a diminished rate, in the presence of a strongly negative nitrogen balance induced by the injection of cortisone acetate (Ingle *et al.*, 1950).

Large Walker carcinomata have been shown to contain more nitrogen than was stored by their hosts during the course of tumor growth when they ate freely a semisynthetic diet that was adequate to maintain growth, pregnancy and lactation in normal rats (Mider *et al.* 1948). Comparable data were also obtained from a study of the Murphy-Sturm rat lymphoma (Mider, 1951). The contributions of ingested nitrogen to

the metabolic pool may be adequate to supply the necessary building blocks for both host and tumor until the tumor reaches approximately 10% of the total body weight (rat plus tumor), but larger lymphomas contain more nitrogen than could have been derived from the diet alone. A similar situation may be inferred from the data for the Walker carcinoma 256 presented by Mider *et al.* (1951).

In a study of the potential sources of tumor nitrogen from the host Sherman *et al.* (1950) found that most of the organs and tissues which lost nitrogen during simple starvation also relinquished nitrogen during progressive growth of the Walker carcinoma 256. The liver and spleen gained nitrogen temporarily but sometimes lost it terminally. In none of the work by Mider and his co-workers has any consistent significant difference between the urinary and fecal excretion of nitrogen by rats bearing Walker carcinoma 256 and pair-fed noncancerous rats of the same sex and initial body weight been observed during the greater part of tumor growth if the rats ate freely a semisynthetic diet adequate for growth, pregnancy, and lactation in normal rats. From their work it would appear that nitrogen lost from body tissues was translocated to the neoplasm. The technics used suggest potential rather than known sources of building materials for the cancerous cells since they measure net change rather than stepwise alterations. Rats bearing Walker carcinoma 256 which were force-fed at a level of 70 cal. a day retained more nitrogen than did their force-fed controls (Begg and Dickinson, 1951). No loss of carcass weight occurred in these animals as compared to the controls when the tumors represented approximately 20 to 24% of the total body weight. No data of the nitrogen content of the carcasses are given in their paper, but it would appear that if enough energy is available and adequate building blocks are supplied the translocation of nitrogen can be prevented during certain stages of tumor growth. Stewart (1952) extended the work of Begg and Dickinson and was unable to prevent loss of carcass weight giving 70 cal. a day to rats bearing Walker carcinoma when the animals were allowed to survive for a sufficiently long period.

Norberg and Greenberg (1951) injected carboxyl-labeled glycine C^{14} intravenously into C_3H mice bearing transplanted Gardner lymphosarcoma. The specific activities of various tissues and the plasma were determined at intervals up to 48 hours and compared with those of the same source in noncancerous mice of the same strain. The cancerous mice incorporated more of the isotope into their protein than did normal mice in corresponding intervals. The uptake was lower in the wasting muscles of the tumor-bearing mice than in the muscle of normal mice. These findings suggest an increased protein turnover in cancerous mice among those parts of the body which are concerned with protein transport and

synthesis with muscle serving as a major protein source to supplement dietary intake. Recently Tyner *et al.* (1952) have shown that while the total specific activity of liver and kidney protein of cancerous rats receiving food plus isotopically labeled glycine diminished with time, the total activity of the tumor did not. LePage *et al.* (1952) have shown that the total C^{14} increases in the tumors (Flexner-Jobling) of rats fed 2-C^{14}-glycine whether the animals are fed or fasted and that under fasting conditions little of the 2-C^{14}-glycine goes to the noncancerous tissues. It would appear that although the noncancerous tissues maintain a dynamic interchange with the metabolic pool, incorporation of amino acids into tumor is essentially a one-way passage from the metabolic pool to the tumor. This confirms the concept of the tumor as a nitrogen "trap" postulated by Mider *et al.* (1948) on the basis of nitrogen balance studies, carcass and tumor analyses of rats bearing Walker carcinoma 256.

V. Summary

Data derived both from spontaneous tumors in man and transplanted tumors in animals indicate that nitrogen is stored during tumor growth as long as the dietary intake is high enough and the body fat stores great enough to supply the demands of the host and the tumor for energy. The tumor, however, may continue to grow even in the presence of a negative nitrogen balance.

Studies in man and animals using the balance technic have demonstrated that when the dietary intake becomes inadequate, nitrogen is relinquished by the host and retained by the tumor. This has been conclusively demonstrated by the use of isotopically labeled amino acids, and the presence of a one-way passage of major materials from the metabolic pool to the tumor during progressive growth is now established.

There is some suggestion from metabolic balance studies in man that during the regression of a tumor under certain circumstances the nitrogen which is released is reutilized by the host to replenish his depleted protein stores. This needs confirmation and would be most readily approached in animals using isotopically labeled compounds.

VI. Conclusion

Certain pertinent questions arise concerning the relationship of energy and nitrogen metabolism in the cancerous host and their deviation from normal. Why is the energy expenditure increased? Why is the cancerous subject unable to adjust his metabolic processes to a reduced dietary intake as a normal animal would under similar circumstances? What are the properties of malignant cells which permit them to synthesize protein at the expense of the host? Why is the tumor essentially

a nitrogen trap? Can the nitrogen released from the tumor during regression be utilized by the host to rebuild protoplasm? There are no satisfactory answers to these questions at present, but the evidence which we have suggests certain possibilities.

High rates of anaerobic and aerobic glycolysis are characteristic of most cancer tissues *in vitro* (Warburg, 1930; Greenstein, 1947). Evidence exists that glycolysis also occurs at a high rate in tumors *in vivo*. Efferent blood from the tumors studied *in vivo* by the Coris (1925) contained less glucose and more lactic acid than did the afferent blood. Voegtlin *et al.* (1935) showed that the pH of a tumor was lowered by the intravenous administration of glucose to the tumor-bearing host. Since acidosis is not detectable in the cancerous animal, the lactic acid produced must be metabolized, presumably by the liver for the most part. Although the total energy produced by the oxidation of glucose to carbon dioxide and water must be the same no matter what intermediate steps occur, this does not mean that all the energy is necessarily available to the organism for its metabolic processes. If the lactic acid is oxidized to pyruvate and then oxidized through the tricarboxylic acid cycle, the total energy obtained can be utilized. If, however, the lactic acid is synthesized to glycogen, high-energy phosphate must be utilized in exchange for lower-energy bonds. This represents a wasteful process and may play an important role in the excessive expenditure of energy by the cancerous host in the occasional situation in which the neoplasm makes up a very large proportion of the total metabolically active mass of tissue. It seems unlikely, however, that this represents a major source of energy loss in patients who have relatively small tumors, yet some of these patients have an increased energy expenditure.

Synthesis of new protoplasm requires energy. The rate of growth of neoplasms may exceed that of some normal embryonic tissues. A diurnal variation in the metabolic activity of normal tissue probably exists. Energy studies in man suggest that growth of a malignant neoplasm proceeds throughout the entire day, perhaps at the same rate. If this be so, the metabolic machine, the host, is deprived of the periodic reduction in caloric expenditure which occurs in normal subjects.

The cancerous subject is forced by the presence of the tumor to synthesize new protoplasm. As long as the dietary intake remains adequate, this synthesis can apparently occur without too great cost to the host, even though the capacity of the adult to form new tissue may be less than that of the immature organism. However, as the dietary intake decreases, the demands on the host seem to increase with progressive growth of the neoplasm, since the ingested food is no longer adequate to supply the needs of both. The metabolic pattern of the host must be considerably

altered when the building blocks used by the neoplasm are derived from endogenous rather than exogenous sources and progressive growth of the tumor becomes increasingly expensive in terms of energy and nitrogen metabolism. *In vitro* experiments suggest that several different mechanisms exist by which the body may build protoplasm. There is probably some diversity in the manner in which the necessary energy may be obtained. Decrease in alimentation in the presence of a demand for continuing synthesis of protoplasm may force the organism into pathways of intermediary metabolism more expensive than those that the organism would use were the usual plethora of fuel and building blocks available to it. The mechanism of anorexia in cancer is completely unknown and deserves intensive investigation at both experimental and clinical levels.

No qualitative differences have been demonstrated between the enzymes of tumor tissue and those of normal tissue. However, there are pronounced quantitative differences between them. These may be of such magnitude that the net effect is qualitative. It appears that hepatoma cells have a greater capacity to concentrate glycine across the cell membrane than do normal liver cells (Zamecnik and Frantz, 1949). This capacity may include all amino acids and may represent an increased ability of tumor cells to convert amino acids to proteins. The activity of certain neoplastic cells, at least, seems to be oriented toward the synthesis of proteins rather than the storage of energy-rich materials such as glycogen (Zamecnik *et al.*, 1951). With progressive tumor growth, the amino acids would be removed from the circulating fluid by the neoplastic tissue at a more rapid rate than they would by normal cells, and the effect would be that of a "nitrogen trap." The dynamic equilibrium between normal tissues and the metabolic pool has been amply demonstrated (Schoenheimer, 1942; Shemin and Rittenberg, 1944), but the mechanisms by which amino acids are exchanged between the metabolic pool, normal tissues and neoplastic cells and the stimulus provided by the tumor which results in the liberation of protein, in whatever form, from the tissues of the host so that it may be used by the tumor remain unsolved. Although the blood supply to the tumor may be a factor in preventing the protein fragments which are liberated during necrosis of the central tumor mass from returning to the metabolic pool, other mechanisms should be sought. Perhaps some of the fragments are utilized by the viable, growing neoplastic cells, but a portion is certainly retained in the necrotic areas of the tumor. This does not occur in non-neoplastic tissues that have undergone necrosis and in which healing takes place.

The question of utilization of protein fragments of neoplastic cells which have been freed during spontaneous or therapeutically induced regression of a cancer by that host has not been answered. There is

indirect evidence from balance studies in cancerous patients that nitrogen derived from a regressing lymphosarcoma was utilized by the host to replenish depleted protein reserves (Fenninger and Waterhouse, 1952: Fenninger *et al.*, 1953) but this must be verified by further studies which will probably have to be done in experimental animals. This phenomenon in a sense represents the reversal of the nitrogen "trap." Any light shed on the intermediate steps of either of these phenomena is of the greatest significance in a fundamental understanding of the neoplastic process and will be of considerable importance in the management of clinical neoplastic disease.

It is evident that many of the problems concerning nitrogen and energy metabolism in the host-tumor relationship can be attacked only in the intact animal. *In vitro* studies of tumors have been immensely valuable in demonstrating the possible pathways in the intermediate metabolism of some of the substances which are utilized by neoplasms in their growth. They do not reveal which of these pathways are actually followed in the living cancerous subject. New technics are required for the elucidation of the intermediate steps of energy and nitrogen transfer in normal and in tumor-bearing hosts, and it seems worth while to direct investigative effort in these directions.

REFERENCES

Abels, J. C., Rekers, P. E., Binkley, G. E., Pack, G. T., and Rhoads, C. P. 1942. *Ann. Internal Med.* **16,** 221–240.

Adams, E. 1950. *Proc. Soc. Exptl. Biol. Med.* **75,** 282–285.

Adams, W. S., Alling, E. L., and Lawrence, J. S. 1949. *Am. J. Med.* **6,** 141–161.

Adams, W. S., Valentine, W. N., Bassett, S. H., and Lawrence, J. S. 1952. *J. Lab. Clin. Med.* **39,** 570–581.

Adams, E., and White, A. 1950. *Proc. Soc. Exptl. Biol. Med.* **75,** 590–595.

Albright, F., Bartter, F. C., and Forbes, A. P. 1949. *Trans. Assoc. Am. Physicians* **62,** 204–213.

Albright, F., Forbes, A. P., Bartter, F. C., Reifenstein, E. C., Jr., Bryant, D., Cox, L. D., and Dempsey, E. F. 1950. *In* Symposia on Nutrition of Robert Gould Research Foundation, Vol. II, pp. 155–194. Charles C Thomas, Springfield, Ill.

Albright, F., Forbes, A. P., and Reifenstein, E. C., Jr. 1946. *Trans. Assoc. Am. Physicians* **59,** 221–234.

Ariel, I. M. 1949. *Surg. Gynecol. Obstet.* **88,** 185–195.

Ariel, I. M., Rekers, P. E., Pack, G. T., and Rhoads, C. P. 1943. *Ann. Surg.* **118,** 366–371.

Ariel, I. M., and Shahon, D. B. 1950. *Cancer* **3,** 608–623.

Balch, H. H. 1950. *J. Immunol.* **64,** 397–410.

Ball, H. A., and Samuels, L. T. 1938. *Am. J. Cancer* **32,** 50–56.

Barrett, M. K. 1946. *J. Natl. Cancer Inst.* **7,** 127–157.

Begg, R. W., and Dickinson, T. E. 1951. *Cancer Research* **11,** 409–412.

Benedict, F. G., Miles, W. R., Roth, P., and Smith, H. M. 1919. *Carnegie Inst. Wash. Publ.* No. **280,** 490–525.

Bischoff, F., and Long, M. L. 1938. *Am. J. Cancer* **32,** 418–421.
Bischoff, F., Long, M. L., and Maxwell, L. C. 1935. *Am. J. Cancer* **24,** 549–553.
Boothby, W. M., and Sandiford, T. 1922. *J. Biol. Chem.* **54,** 783–803.
Buckman, T. E., Daland, G. A., and Weld, M. 1925. *Arch. Internal Med.* **35,** 389–401.
Cannon, P. R. 1950. *In* Symposia on Nutrition of the Robert Gould Research Foundation, Vol. II, pp. 312–333. Charles C Thomas, Springfield, Ill.
Collins, D. H., and Rose, W. M. 1948. *J. Pathol. Bacteriol.* **60,** 63–74.
Commons, R. R., and Strauss, M. B. 1948. *Am. J. Med. Sci.* **215,** 525–529.
Cori, C. F., and Cori, G. T. 1925. *J. Biol. Chem.* **65,** 397–405.
Cramer, W., and Pringle, H. 1910. *Proc. Roy. Soc.* (*London*) **B82,** 307–320.
Cuthbertson, D. P., and Tompsett, S. L. 1935. *Brit. J. Exptl. Pathol.* **16,** 471–475.
Dent, C. E., and Rose, G. A. 1949. *Biochem. J.* **44,** 610–618.
Dillard, H. L., Pearsall, H. R., and Chanutin, A. 1949. *Cancer Research* **9,** 661–664.
Dubin, I. N. 1947. *Ann. Internal Med.* **27,** 898–913.
Drummond, J. C. 1916. *Biochem. J.* **10,** 77–88.
Drummond, J. C. 1917. *Biochem. J.* **11,** 325–377.
Eckhardt, R. D., and Davidson, C. S. 1950. *In* Symposia on Nutrition of the Robert Gould Research Foundation, Vol. II, pp. 275–311. Charles C Thomas, Springfield, Ill.
Eckhardt, R. D., Lewis, J. H., Murphy, T. L., Batchelor, W. H., and Davidson, C. S. 1948. *J. Clin. Invest.* **27,** 119–134.
Eliel, L. P., Pearson, O. H., Katz, B., and Kraintz, F. W. 1950. *Federation Proc.* **9,** 168.
Fenninger, L. D., and Waterhouse, C. 1951. *Cancer Research* **11,** 247.
Fenninger, L. D., and Waterhouse, C. 1952. *Proc. 2nd Natl. Cancer Conf.* (In press.)
Fenninger, L. D., Waterhouse, C., and Keutmann, E. H. 1953. *Cancer* **6,** 930–941.
Forkner, C. E. 1938. Leukemia and Allied Disorders. The Macmillan Co., New York.
Gimbel, N. S., Rugel, C., and Glenn, W. W. L. 1950. *J. Clin. Invest.* **29,** 998–1009.
Greenstein, J. P. 1947. The Biochemistry of Cancer. Academic Press, New York.
Gunderson, A. H. 1921. *Boston Med. Surg. J.* **185,** 785–787.
Haven, F. L., Bloor, W. R., and Randall, C. 1949. *Cancer Research* **9,** 511–514.
Haven, F. L., Bloor, W. R., and Randall, C. 1951. *Cancer Research* **11,** 619–623.
Heindl, A., and Trauner, R. 1927. *Mitt. Med. u. chir.* **40,** 416–432.
Henderson, Y., and Edwards, G. H. 1903. *Am. J. Physiol.* **9,** 417–424.
Huggins, C. 1949. *Cancer Research* **9,** 321–327.
Ingle, D. J., Prestrud, M. C., Li, C. H., and Evans, H. M. 1947. *Endocrinology* **41,** 170–176.
Ingle, D. J., Prestrud, M. C., and Rice, K. L. 1950. *Endocrinology* **46,** 510–513.
Jaffe, R. H. 1933. *Folia Hematologica* **49,** 51–63.
Jaffe, R. H. 1935. *Arch. Pathol.* **20,** 725–741.
Keys, A., Brozek, J., Henschel, A., Mickelsen, O., and Taylor, H. L. 1950. The Biology of Human Starvation. University of Minnesota Press, Minneapolis, Minn.
Kinsell, L. W., Margen, S., Tarver, H., Frantz, J. M., Flanagan, E. K., Hutchin, M. E., Michaels, G. D., and McCallie, D. P. 1950. *J. Clin. Invest.* **29,** 238–250.
Klatskin, G., and Rappaport, E. M. 1947. *Ann. Internal Med.* **26,** 13–26.
Knudson, A., and Erdos, T. 1917. *Boston Med. Surg. J.* **176,** 503–507.
Königer, H. 1906. *Deut. Arch. klin. Med.* **87,** 31–61.
Lennox, W. G., and Means, J. H. 1923. *Arch. Internal Med.* **32,** 705–708.
Le Page, G. A., Potter, V. R., Busch, H., Heidelberger, C., and Hurlbert, R. B. 1952. *Cancer Research* **12,** 153–157.
Madden, S. C., and Whipple, G. H. 1940. *Physiol. Revs.* **20,** 194–217.

McEuen, C. S., and Thomson, D. L. 1933. *Brit. J. Exptl. Pathol.* **14,** 384–391.
Medigreceanu, F. 1910. *Berlin. klin. Wochschr.* **47,** 772–775.
Mehl, J. W., and Golden, F. 1950. *J. Clin. Invest.* **29,** 1214–1218.
Mider, G. B. 1951. *Cancer Research* **11,** 821–829.
Mider, G. B. 1953. *Ann. Rev. Med.* **4,** 187–198.
Mider, G. B., Alling, E. L., and Morton, J. J. 1950. *Cancer* **3,** 56–65.
Mider, G. B., Fenninger, L. D., Haven, F. L., and Morton, J. J. 1951. *Cancer Research* **11,** 731–736.
Mider, G. B., Sherman, C. D., Jr., and Morton, J. J. 1949. *Cancer Research* **9,** 222–224.
Mider, G. B., Tesluk, H., and Morton, J. J. 1948. *Acta Unio Intern. contra cancrum* **6,** 409–420.
Milroy, T. H., and Malcolm, J. 1898. *J. Physiol.* **23,** 217–239.
Minot, G. R., and Means, J. H. 1924. *Arch. Internal Med.* **33,** 570–580.
Mischtschenko, I. P., and Fomenko, N. M. 1928. *Z. Krebsforsch.* **27,** 427–435.
Moraczewski, W. von. 1898. *Virchow's Arch. pathol. Anat. u. Physiol.* **151,** 22–52.
Moreschi, C. 1909. *Z. Immunitätsforsch* **2,** 651–685.
Müller, F. 1889. *Z. klin. Med.* **16,** 496–549.
Murphy, J. B., Means, J. H., and Aub, J. C. 1917. *Arch. Internal Med.* **19,** 890–907.
Newburgh, L. H. 1942. *Arch. Internal Med.* **70,** 1033–1096.
Norberg, E., and Greenberg, D. M. 1951. *Cancer* **4,** 383–386.
Oppenheim, A., Abels, J. C., Pack, G. T., and Rhoads, C. P. 1945. *J. Am. Med. Assoc.* **127,** 273–276.
Ordway, T. 1919. *J. Am. Med. Assoc.* **73,** 860.
Parfentjev, I. A., Cliffton, E. E., and Duran-Reynals, F. 1951. *Science* **113,** 523–524.
Pearson, O. H., and Eliel, L. P. 1951. *Recent Progr. Hormone Research* **6,** 373–412.
Pearson, O. H., Eliel, L. P., Rawson, R. W., Dobriner, K., and Rhoads, C. P. 1949. *Cancer* **2,** 943–945.
Pearson, O. H., Eliel, L. P., and White, F. C. 1950. *In* Pituitary-Adrenal Function. R. C. Christman (ed.), pp. 145–148. American Association for the Advancement of Science, Washington, D.C.
Petermann, M. L., and Hogness, K. R. 1948. *Cancer* **1,** 100–103.
Petermann, M. L., Karnofsky, D. A., and Hogness, K. R. 1948. *Cancer* **1,** 109–119.
Peters, J. P. 1946. *Ann. N. Y. Acad. Sci.* **47,** 327–344.
Pettenkofer, M., and Voit, C. 1869. *Z. Biol.* **5,** 319–328.
Popper, H., and Schaffner, F. 1950. *Advances in Internal Med.* **4,** 351–443.
Reinfenstein, E. C., Jr., Albright, F., and Wells, S. L. 1945. *J. Clin. Endocrinol.* **5,** 367–395; 1946. *Correction* **6,** 232.
Ross, J. F., Crockett, C. L., Jr., and Emerson, C. P. 1951. *J. Clin. Invest.* **30,** 668–669.
Rous, P. 1914. *J. Exptl. Med.* **20,** 433–451.
Schoenheimer, R. 1942. The Dynamic State of the Body Constituents. Harvard University Press, Cambridge, Mass.
Seibert, F. B., Seibert, M. V., Atno, A. J., and Campbell, H. W. 1947. *J. Clin. Invest.* **26,** 90–102.
Shemin, D., and Rittenberg, O. 1944. *J. Biol. Chem.* **153,** 401–421.
Shen, S. C., and Homburger, F. 1951. *J. Lab. Clin. Med.* **37,** 182–198.
Sherman, C. D., Jr., Morton, J. J., and Mider, G. B. 1950. *Cancer Research* **10,** 374–378.
Stewart, A. G. 1952. Thesis, University of Western Ontario. London, Ontario.
Strieck, F., and Mulholland, H. B. 1928. *Deut. Arch. klin. Med.* **162,** 51–67.
Sturgis, C. C. 1948. Hematology, 1st ed., Charles C Thomas, Springfield, Ill.

Tagnon, A. J., and Trunnell, J. B. 1948. *Cancer* **1,** 472–482.
Tannenbaum, A. 1942. *Cancer Research* **2,** 460–467.
Taylor, H. L., Mickelsen, O., and Keys, A. 1949. *J. Clin. Invest.* **28,** 273–281.
Terry, R., Sandrock, W. E., Nye, R. E., Jr., and Whipple, G. H. 1948. *J. Exptl. Med.* **87,** 547–559.
Tyner, E. P., Heidelberger, C., and Le Page, G. A. 1952. *Cancer Research* **12,** 158–164.
Voegtlin, C. 1937. *Physiol. Revs.* **17,** 92–118.
Voegtlin, C., Fitch, R. H., Kahler, H., Johnson, J. M., and Thompson, J. W. 1935. *Natl. Inst. Health, Bull.* No. **164,** 1–15.
Wallersteiner, E. 1914. *Deut. Arch. klin. Med.* **116,** 145–187.
Wallhauser, A. 1933. *Arch. Pathol.* **16,** 522–562, 672–712.
Warburg, O. 1930. The Metabolism of Tumors. E. Dickens (transl.), Constable, London.
Waterhouse, C., Bassett, S. H., and Holler, J. W. 1949. *J. Clin. Invest.* **28,** 245–264.
Waterhouse, C., Fenninger, L. D., and Keutmann, E. H. 1951. *Cancer* **4,** 500–514.
Whipple, G. H. 1942. *Proc. Inst. Med. (Chicago)* **14,** 2–11.
White, F. R. 1945. *J. Natl. Cancer Inst.* **5,** 265–268.
White, W. H., and Hopkins, F. G. 1899. *J. Physiol.* **24,** 42–47.
Willis, R. A. 1948. Pathology of Tumors, pp. 25–27. C. V. Mosby Co., St. Louis, Mo.
Winzler, R. J., and Smyth, I. M. 1948. *J. Clin. Invest.* **27,** 617–619.
Zamecnik, P. C., and Frantz, I. D., Jr. 1949. *Cold Spring Harbor Symposia Quant. Biol.* **14,** 199–208.
Zamecnik, P. C., Loftfield, R. B., Stephenson, M. L., and Steele, J. M. 1951. *Cancer Research* **11,** 592–602.
Zeldis, L. J., and Alling, E. L. 1945. *J. Exptl. Med.* **81,** 515–537.
Zeldis, L. J., Alling, E. L., McCoord, A. B., and Kulka, J. P. 1945. *J. Exptl. Med.* **82,** 157–179.

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Some Aspects of the Clinical Use of Nitrogen Mustards

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CONTENTS

I. INTRODUCTION

Considerable information has been accumulated on the properties and the pharmacological actions of the nitrogen mustards. The original impetus for study, namely the potential wartime usefulness of these compounds, was greatly increased by the discovery of their radiomimetic properties and the value of certain of them as cancer chemotherapeutic agents. As a result, in addition to the many original contributions, comprehensive reviews have been published. The latter deal with the chemistry (Achard, 1919), pharmacology (Gilman, 1946), and use of the nitrogen mustards in cancer chemotherapy (Karnofsky, 1950). Most data in the more basic reviews are derived from the *in vitro* or animal studies. Most information in oncological reviews refers to the effect on the tumor of a "standard" course of therapy usually with methyl bis(β-chloroethyl)amine.*

The mustard first synthesized was sulfur mustard. This was done in 1860 by Guthrie, who first noted its vesicant action. In 1887, Meyer described the necrotizing effect of this agent on the skin, respiratory tract

* Hereinafter referred to as HN_2.

and eyes. Thus, mustard gas or sulfur mustard differs from most other suffocating gases in that its effects are not confined to the respiratory tract. Krumbhaar (1919a,b, 1919–20) perhaps first appreciated the extent to which the hematopoietic system is affected by these compounds. This concept, however, was not at first generally accepted (Moorehead, 1919; Warthin and Weller, 1919).

The development of agents which could be handled with reasonable ease and safety was required before extensive clinical studies could be done. Adair and Bagg in 1931 made an attempt to use vesicant vaporized mustard as a cancer chemotherapeutic agent, but they abandoned the studies because of the technical difficulties in handling the agent.

The first compound of the present nitrogen mustard series, tris(2-chloroethyl)amine, was prepared in 1935 by Ward. Many related compounds have since been synthesized, but extensive clinical trial has been limited almost exclusively to HN_2. This compound, in combination with hydrochloric acid, forms a stable white powder which is soluble in water and quite easy to handle.

This presentation will be a discussion of the action of HN_2 on human patients. Effects on the tumors and other disease entities which were present in these patients will be given secondary consideration. Animal and *in vitro* work will be quoted only to support clinical observations. Considerable data from and observations made by the authors on a large series of cancer patients who received varying but often large total doses of HN_2 will be included. The effect of the drug will be discussed primarily by system and organ.

Clinical effects produced by administration of HN_2 vary according to dosage schedule used. Based on the results of the original experimental work on the effects of the drug on mice with lymphosarcoma, the first patients treated with HN_2 compounds received 0.1 mg./kg. body weight on each of 10 successive days (Craver, 1948). This dose proved to be too great. Although marked regression of radioresistant lymphosarcoma was produced, severe toxic effects were also noted. A smaller dose was then used and, subsequently, the present standard course (0.1 mg./kg. body weight daily for 4 days) was adopted on the basis that this amount of HN_2 produced a remission of certain tumors for an average of 2.2 months, whereas it produced a depression of bone marrow for an average of only 1.2 months (Spurr *et al.*, 1948). Minor variations in the size of the daily intravenous dose of HN_2 have been used but have shown no increased therapeutic effect (Apthomas *et al.*, 1947; Karnofsky *et al.*, 1948a; Kreiner and Bauer, 1951; Lynch *et al.*, 1950; Wintrobe and Huguley, 1948). Use of small individual doses permits the administration of a larger total dose. Periodic injections in an attempt at maintenance therapy in the treatment

of lymphomas has been successfully used over an extended period of time (Wintrobe and Huguley, 1948).

HN_2 has radiomimetic properties which, like those of other sulfur and nitrogen mustards, are related to the inhibition of mitosis, an action which is nicely measured by noting the effect of the locally applied drug on the cells of the cornea of the rat (Friedenwald *et al.*, 1947). By this method, microscopic evidence of mitotic inhibition was produced with doses of HN_2 proportionally far smaller than those required to produce clinically recognizable effects. This inhibition of mitosis could be made to persist several weeks by repeated instillation of HN_2. With these experimental data in mind, plus the known radiomimetic properties of mustard, an attempt was made to duplicate as closely as possible the successful methods of administering roentgen rays in the treatment of cancer. Patients were treated with frequent small doses of HN_2 administered over a prolonged period of time by intra-arterial injection into local areas (Bateman *et al.*, 1951; Cromer *et al.*, 1952; Klopp *et al.*, 1950a,b), duplicating as near as possible the use of fractionated local x-ray therapy. By this method, increased local effects were noted. However, unless very small individual doses were given, the average total dose which could be administered was not much greater than that which would have been tolerated if the drug had been administered intravenously. Specific exceptions to this rule were noted and will be discussed later.

Like variation in dose schedule, route of administration may influence the effect produced on individual organs, the total organism and the tumor, where present. This is clearly demonstrated by the fact that vesiculation of the skin is produced by local application but never by intravenous administration. Adair and Bagg (1931) noted that local application of mustard vapor to, or injection of it into cancer tissue was followed by regression of the tumor, an effect which could be produced by no other method of administration. Similarly, injection of HN_2 into the pleural cavity is followed by a more marked decrease in the rate of accumulation of fluid associated with the presence of implants of tumor on pleural surfaces than is seen following systemic administration of the same drug (Karnofsky *et al.*, 1948a).

The site of intravascular injection also modifies the response. As a generalization, the intra-arterial injection of HN_2 either in a single massive dose (Bierman *et al.*, 1950a,b, 1951a,b) or in multiple small doses (Bateman *et al.*, 1951; Cromer *et al.*, 1952; Klopp *et al.*, 1950a,b) produces in the regional area effects which will not be seen following the intravenous administration of maximum tolerated amounts of the drug. Methods for the injection of massive single doses of HN_2 into specific arteries (Bierman *et al.*, 1950a) and for the insertion of an indwelling cannula into

the regional artery have been described (Klopp *et al.*, 1950a). The latter method makes feasible the periodic injection of drugs into an artery over an extended period of time.

Changes in physical factors modify the effect of HN_2 on specific organs or cells. For instance, the blood supply of not only some cancers (Algire and Chalkley, 1945; Bierman *et al.*, 1951c) but also of certain organs is richer than that of others. Following the injection of a drug into the vascular system, the amount which will be delivered to a given organ or tissue will be determined, at least in part, by the amount of blood delivered to the organ or cells in question. It is well known that most malignant tumors encourage the local development of increased blood supply (Bierman *et al.*, 1951b), and this increased vascularity may be partially responsible for the efficacy of HN_2 as a cancer chemotherapeutic agent. Similarly, the fibrosis and decrease in blood supply produced by roentgen ray therapy may decrease the relative amount of HN_2 delivered to the cancer and be responsible for the poorer therapeutic effect noted in the treatment of previously irradiated lymphomata (Alpert *et al.*, 1950). Whether similar reasoning applies to organs cannot be stated.

Presumably, certain cells will detoxify HN_2 or remove it more rapidly from the blood stream than will others. The presence of specific cells within the capillary bed just beyond the injection site should, therefore, influence the amount of HN_2 made available to body tissue elsewhere. The specific cells which receive the initial impact of the drug may be components of a special organ such as liver or a tumor. Adair and Bagg (1931) noted that the presence of malignant tumor seemed to afford some degree of protection against the effect of a large dose of mustard gas. The presence of a large malignant tumor within the regional tissue into which a close intra-arterial injection has been made can be correlated with safe administration of a larger total dose of HN_2 (Bateman *et al.*, 1951) than is tolerated when only a small mass of cancer tissue is present.

Pooling of blood within a regional area may increase toleration to HN_2. For instance, if the venous return from the treated region is occluded during and for five minutes after the intra-arterial administration of HN_2, the total dose of the agent which may be administered into that regional area can be increased over that tolerated when venous channels are open (Bateman *et al.*, 1951). This has been demonstrated in the dog following the close intra-arterial injection of radioactive gold (Berg, 1951). In the latter studies, when the afferent injected artery and efferent vein to a region were both occluded during the time the radioactive gold was injected, a tremendously increased concentration of gold was deposited in the intervening peripheral capillary bed as measured by the radioactivity of the region. During studies of the value of the intra-arterial injection of

HN_2 for therapy of regional cancer, it was noted that those patients in whom the afferent veins from the regionally treated area had been previously ligated or were compressed by tumor tolerated a larger total dose than those in whom the efferent venous system was patent (Bateman *et al.*, 1951).

Regional arterial occlusion may influence results in other ways. If an arterial branch distal to the site of injection is occluded during the time of an intra-arterial HN_2 injection, no peripheral effect will appear distal to the site of occlusion (Klopp *et al.*, 1950a). If the arterial supply to the intestine is occluded during and for 5 to 10 minutes following intravenous injection of HN_2 into rats, microscopic changes in the epithelium of the intestinal tract will not be noted in the region distal to the site of occlusion (Karnofsky *et al.*, 1948b). Thus, if the arterial supply to a given region of the body is occluded during and for a short period of time following the injection of HN_2, the regional tissue just beyond the site of occlusion will be protected. This protection affords increased drug tolerance. Complete exclusion of bone marrow by some method would be desirable but no such method has been devised; however, the effectiveness of regionally injected HN_2 has been enhanced by selective arterial occlusion. For example, blood pressure cuffs, applied to both thighs, were inflated sufficiently to obliterate the arteries of the lower extremities during and for a short period following injection into the lower abdominal aorta. The volume of the vascular bed available to the first wave of HN_2 was greatly decreased and local concentration of the drug in the pelvis was achieved (Cromer *et al.*, 1952).

By use of vasoconstricting agents, selective partial arterial occlusion may also be possible, particularly in relation to masses of cancer tissue as the latter have been demonstrated to have arteries which show a minimal, if any, response to the constricting action of circulating epinephrine (Bierman *et al.*, 1951a). The intravascular injection of epinephrine might be expected to result in the usual constriction of arteries to most normal tissue but not of those supplying the cancer mass. The intravascular injection of HN_2 at this moment would result in the delivery to the tumor of an increased percentage of the first wave of the intravascularly administered HN_2 (Bierman *et al.*, 1951a). As yet, there is no decisive evidence that this theoretical concept is of practical value.

The solvent for the HN_2 may be important. A solution of mustard gas in propylene glycol caused only diffuse pulmonary congestion following intravenous injection in animals, whereas when administered undiluted it produced severe necrotizing pulmonary lesions (Anslow, 1948). On the other hand, HN_2 when dissolved in saline for routine therapy produced no clinically significant pulmonary lesions, although some

microscopic, intranuclear changes were found in the lungs of small animals (Skipper *et al.*, 1951).

In the following sections, effects produced by administration of HN_2 will be discussed by organ, system, or in terms of general metabolic effect.

II. Skin and Appendages

Like sulfur mustard, HN_2 when applied directly has a vesicant necrotizing action on skin. The severity is roughly dependent upon the concentration of the applied drug and the duration of contact with the skin. When the concentration of applied HN_2 is sufficiently great and/or the period of contact is of sufficient duration, epilation of hair is produced at the site of application. That production of epilation depends on concentration delivered to the cells of the skin is illustrated by a comparison of the results following intravenous therapy with those following intra-arterial therapy of certain hair-bearing regions. No epilation results from a routine course of intravenously administered HN_2, while complete localized loss of hair occurs within the skin area supplied by the capillary bed immediately distal to the site of intra-arterial injection of the same amount of the HN_2. This localized epilation occurs only when the volume of the injected arterial tree is relatively small. Unilateral loss of pubic hair has been noted following injection into a common iliac artery, whereas no loss of pubic hair has been seen following injection into the abdominal aorta. Epilation also depends on the type of hair. It has never been noted in the hair of the eyebrows or extremities. It always occurs when beard and scalp areas have been treated and is only seen in the pubic region when therapy has been unilateral. This variation in response in the end organ appears to be related not only to concentration delivered but also to the degree of activity of treated hair follicles, the effect being most intense in areas where hair growth is rapid and sustained.

Skin necrosis, like epilation, cannot be produced by intravenous HN_2 therapy, but can result from direct application and by the administration of a massive single dose into the artery supplying a skin area (Bierman *et al.*, 1951b). Administration of smaller doses of HN_2 into the same artery produces merely a delayed erythema first seen in 7 to 10 days. This slowly fades and is followed by a dry scaling of the skin and, occasionally, by mild pigmentation. The skin necrosis which follows the intra-arterial administration of a single massive dose is associated with the nonspecific destruction of all exposed cells. The changes produced by administration of smaller doses are related to antimitotic action of HN_2 on the more rapidly dividing cells of the basilar layers of the epithelium. The deep penetration of this agent has been demonstrated

following the local application of radioactive sulfur mustard to skin (Axelrod and Hamilton, 1947), and perhaps explains the depth of the tissue destruction which results from the local application of high concentrations of HN_2. The destruction and necrosis produced by the application of high concentrations of HN_2, being nonspecific, affect blood vessels and decrease the vascularity of the region. The local anemia which is produced is largely responsible for the slow healing. Erythema resulting from administration of lesser doses of HN_2 is slow to develop and is attributed to the reaction to destroyed cells, or to denatured material, or to the drug accumulated in interstitial spaces. The latent period is too long to explain the development of erythema entirely on the basis of vasodilatation due to the direct action of HN_2.

III. Plasma

HN_2 reacts quickly with many substances. Following intravascular injection, the compound undergoes intramolecular cyclization in the polar solvent, plasma, to form a cyclic ethylenimonium compound (Gilman, 1946). It has been postulated that, by this mechanism, the drug is rapidly inactivated and detoxified either while still within the blood stream or shortly after reaching the tissues. For example, in experimental animals, the intestinal epithelium could be protected by occluding the arterial blood supply for 10 to 20 minutes during and immediately following the injection of HN_2 (Karnofsky *et al.*, 1948b). Similarly in human patients epilation of the scalp produced by the intra-arterial administration of HN_2 could be prevented by occluding the artery distal to the injection site (Klopp *et al.*, 1950a). These effects may be attributed more to a decrease in the initial concentration of the drug delivered to the cell than to rapid detoxification. Such a concept is supported by the facts that HN_2 when tested against embryonic heart muscle in tissue culture is found to exert a toxic effect over a period of at least 48 hours (Cornman), and that when administered intravascularly in sufficiently small amounts and at a slow rate (1 mg. every 12 hours) it will not produce any significant bone marrow depression even though the total amount administered is in excess of that known to produce such changes when administered in larger amounts at a more rapid rate (Bateman *et al.*, 1951).

Intravascularly administered HN_2 is retained in the blood stream for a short period of time, as measured by studies of rate of disappearance of related mustards tagged with I^{131} (Seligman *et al.*, 1950). The drug is well distributed in peripheral tissues within a matter of minutes. A slightly increased amount is deposited in the first capillary bed. This is demonstrated by the intravenous administration of C^{14}-labeled HN_2,

following which there is an increased tissue concentration present in lung as compared to that present in most other tissue (Skipper *et al.*, 1951).

Serial x-rays taken following injection of thorotrast into the iliac artery of a dog demonstrated a delayed appearance of a fine network of radiopaque material in the thigh. Such evidence suggested that not all of the thorotrast returned by the venous route but that some was trapped in lymph channels. Lymph flow from the skin of dogs contaminated with liquid mustard gas increases. Lymph collected from vessels draining the contaminated area inhibits the growth of bone marrow fragments in tissue culture, indicating the presence of mustard gas or a toxic derivative (Cameron and Courtice, 1948).

The fate of interstitial or intracellularly deposited HN_2 is not known. HN_2 must traverse the interstitial space to enter tissue cells, and it is probable that a certain amount within the intercellular fluid is carried into the lymph channels, thence through lymph nodes and major lymph channels back into the vascular tree. A delayed increase in the concentration of HN_2 in the lymph nodes would be expected, as they would have received the HN_2 from two sources at different times, namely the arteries and the afferent lymphatic channels of the lymph node. A delayed increase within lymph nodes has been noted in animals following the administration of C^{14}-labeled HN_2 (Skipper *et al.*, 1951). Clinical experience also furnishes suggestive evidence of an increased effect on lymph node tumors as compared to other cancers, and a more pronounced effect on the cells of the lymph nodes as compared to testicular cells which are considered equally sensitive.

The actual manner in which HN_2 is transported within the blood and other body fluids is not known. Therapeutic doses of HN_2 do not initiate or aggravate any abnormalities in blood chemistry as measured by estimations of concentrations of total protein, serum albumin and globulin, nonprotein nitrogen, and amino acid nitrogen. The only substance whose blood concentration is known to be rapidly altered (decreased) by the intravascular administration of HN_2 is vitamin A. Electrolyte changes which have been noted have followed a prolonged period of administration of HN_2. These are a depression of serum sodium and, in some cases, slight elevation of serum potassium.

IV. Hematopoietic System

Early observations of the hematologic changes occurring as a result of sulfur mustard gas poisoning were variable. Moorehead (1919) and Warthin and Weller (1919) both reported that a leukocytosis was produced. The latter had noted the development of an anemia and an occasional decrease in circulating platelets, whereas the former had

observed an increase in hemoglobin and red blood cell count. Krumbhaar's (1919a, 1919–1920) observations on victims of mustard gas poisoning were similar to those now seen following therapeutic administration of HN_2, namely an initial leukocytosis followed by a degree of leukopenia and anemia dependent on severity of exposure or size of dose. In the Bari harbor casualties of 1947, leukopenia developed (Alexander, 1947). In some, hemoconcentration also developed which could be attributed not to the toxic effects of mustard itself but to the associated shock. A similar explanation would readily explain the increase in hemoglobin and red cell count reported by Moorehead (1919).

The initial effect on the circulating white blood cells of the administration of a moderately large dose of HN_2 is leukocytosis. Leukopenia follows. The degree of these responses, as well as the timing, depends on the size of the individual dose, the size of the total dose, and the site of injection. The standard course of therapy (0.1 mg./kg. body weight daily for 4 days given by the intravenous route) usually produces a leukocytosis for 2 to 3 days, followed by a leukopenia (2 to 5000 total white blood cell count) in 7 to 10 days, with complete recovery in two weeks (Karnofsky, 1950). The same daily dose given intravenously for 10 days results in severe granulopenia and thrombocytopenia (Craver, 1948). A smaller dose (1 mg.) given into a carotid artery at intervals of 8 to 12 hours produced no leukopenia even though administration was continued until a total of 0.6 mg./kg. body weight had been given (Klopp *et al.*, 1950a). A dose of 2 mg. given at the same intervals and by the same route resulted in leukopenia, but the total dose required for this effect was greater than that needed to produce the same degree of depression when the drug was administered intravenously (Klopp *et al.*, 1950a).

The much longer life span of the red blood cells as compared to the white blood cells accounts for the lack of immediate change in the former. Following a therapeutic course of HN_2, a delayed anemia can occur as the peripheral manifestation of the histologically proven early depression of the red cell precursors in the bone marrow. This depression is similar to that exerted on the granulocyte precursors, but unlike that noted on leukocytes, peripheral reaction of the red cells to this central bone marrow change cannot be noted until the life span of the circulating erythrocytes has been exceeded (Bateman *et al.*, 1951).

The site of intravascular injection influences the effect on the hematopoietic system. Administration of a single large dose of HN_2 into the hepatic artery produces a lesser degree of leukopenia than results from intravenous injection of the same amount of drug (Bierman *et al.*, 1951a). Repeated small (1 mg.) injections of HN_2 given into the hepatic artery

produced no evidence of bone marrow depression or leukopenia until more than 70 mg. had been administered. This evidence suggests that the liver cells either detoxify the HN_2 more rapidly or retain a much greater proportion of the drug than do other cells. A similar absence of bone marrow depression was noted following the administration of 125 mg. of HN_2 into the femoral artery supplying a leg which was the site of an enormous fibrosarcoma, suggesting that the cells of the malignant tumor acted in a manner similar to the liver cells (Klopp *et al.*, 1950a).

Thrombocytopenia has been reported following routine HN_2 therapy of certain lymphomas (Bauer and Erf, 1950; Smith *et al.*, 1948; Taffel, 1947; Wawro, 1948) and following the administration of a large total dose of HN_2 (1 mg./kg. body weight within 10 days) (Bateman *et al.*, 1951). A clinically significant degree of thrombocytopenia has been seen once following fractionated intra-arterial HN_2 therapy, and then only after a large total dose had been administered in a short period of time (63 mg. in 8 days). This, like the associated minimal depression of peripheral leukocytes and distal bone marrow noted in these cases may be related to the presence of a very large mass of cancer tissue within the field supplied by the artery injected.

The cellular changes in the bone marrow resemble those seen in the peripheral blood, but appear earlier. Mature eosinophils disappear rapidly in almost all instances (Bateman *et al.*, 1951). Block and coworkers (1948) described the changes following HN_2 therapy as consisting of a primary cytotoxic phase followed by an atrophic phase lasting from the eighth day until the onset of regeneration on the fifteenth to twentieth day. The atrophic phase was primarily due to the decrease in the neutrophil and eosinophil precursors. Plasma cells never showed a decrease in number. Erythroblasts were slightly decreased or unchanged; the latter observation is not in agreement with that of others who have noted depression of red cell precursors (Bateman *et al.*, 1951). The atrophic phase is followed by a prolonged period of overactivity (Block *et al.*, 1948).

By study of sternal marrow obtained at frequent intervals during a protracted course of HN_2 therapy, it is possible to predict quite accurately the peripheral blood picture which will be present in the next 2 to 4 days. The careful study of bone marrow specimens obtained at periodic intervals during therapy is the most reliable method of determining when a given course of HN_2 must be terminated. Therapy should be discontinued when the cellularity of the marrow has been decreased to one-quarter of normal and when the cells present are predominately mature polymorphonuclear leukocytes (Bateman *et al.*, 1951).

By the third week after a dose of 25 mg. of HN_2, the total count of

nucleated cells in the bone marrow falls from the normal level of around 100,000 cells to about 17,000 per cubic millimeter (Spurr *et al.*, 1948). At this stage, there may already be evidence of regeneration in the cells of the remaining myeloid and erythroid tissue. If a larger dose has been administered (60 mg. in 7 days), the bone marrow has shown, within 30 days, extreme atrophy of the marrow with myxomatous degeneration and the presence of a sprinkling of plasma cells, hematocytoblasts, and inflammatory polyblasts with only an occasional small focus of erythroblasts. Regeneration may still take place. A patient so treated showed at autopsy ten months later, regeneration which was in every way complete and normal (Block *et al.*, 1948). The rate of hematologic recovery in patients without neoplastic involvement of bone marrow may be quite rapid. For example, a patient who had received a total of 1.08 mg./kg. body weight of HN_2 demonstrated, within 10 days, so severe a depression of bone marrow that it was possible to detect only an occasional nucleated cell on repeated sternal marrow aspirations. At the same time, peripheral white blood cells were too few to count. Nine days later, the sternal marrow was completely normal. Three days following sternal marrow examination the peripheral total white blood cell count was 6600.

Severe bone marrow depression may result from a relatively small total dose of HN_2 given to a small individual. Such was the case in a woman with recurrent epidermoid carcinoma of the cervix, cachexia, and transverse myelitis resulting from a large metastatic lesion involving the lumbar spine. This patient received 40 mg. of HN_2 (estimated at 1.28 mg./kg. body weight) in 7 days by the intra-arterial route. The bone marrow was aplastic when the patient died 10 days following completion of therapy (Bateman *et al.*, 1951). This total dose (40 mg.) has produced no such severe bone marrow depression in other patients treated in the same manner. However, this relative dose (1.28 mg./kg. body weight) was not attained in the other patients so treated.

Studies of total urobilinogen excretion during HN_2 therapy are few, but the available data (Spurr *et al.*, 1947) show that an increase occurs during therapy, suggesting that some hemolysis may be produced. This is a possible explanation for the persistent anemia seen in some patients who have received repeated courses of mustard drugs.

V. Respiratory Tract

HN_2 has never been administered as a therapeutic agent by inhalation in vapor form, but it is reasonable to assume that effects produced by such therapy would be similar to those noted in the military personnel exposed to sulfur mustards which were accidentally released in the Bari harbor disaster (Alexander, 1947). In these victims an intense inflam-

matory reaction occurred in the mucous membranes of the oral cavity, pharynx, larynx and trachea. In certain instances it was sufficient to produce superficial bullous blebs and marked associated submucosal edema. A similar but more localized effect was noted following the injection of repeated therapeutic doses of HN_2 into one or both external carotid arteries or branches of the same (59). This reaction can be described as going through several stages. The actual stage developed and the degree of intensity of the reaction in each stage varied with the size of the individual and total dose as well as with the rate of accumulation of the total dose. Initially there was soft swelling of the treated region followed by brawny edema. Superficial vesiculation and changes best described as similar to the "mucositis following irradiation," subsequently appeared. Finally, if sufficient drug had been administered, patchy superficial ulceration and some necrosis were seen. No one area within the oral cavity appeared more sensitive than another. However, none of the observed patients had any gross tonsillar tissue present prior to therapy, so that the effect of these lymphoid structures could not be noted. The lymphoid tissue at the base of the tongue decreased rather rapidly early in the course of therapy.

Unlike its action on other cancers, intravenously administered HN_2 often produces a regression of primary carcinoma of the nasopharynx. This might be explained as due to an inherent difference in the intracellular physiology of this specific cancer cell. It would be reasonable to assume that the response was due to delivery to this tumor of proportionately more HN_2 than is delivered to cancers in other areas. This is supported by the observation that the response of a cancer of the nasopharynx to the increased concentration following intracarotid injection is even more marked than that produced by intravenous therapy with a similar amount (Klopp *et al.*, 1950a). The delivery of the increased amounts of HN_2 to a cancer of the nasopharynx might be related to the presence of the very rich lymphatic network and the many solid lymphoid structures in this region. These drain the interstitial fluid from intercellular spaces of the base of the skull and in so doing may deliver an additonal amount of interstitially deposited HN_2 into the tumor.

The effect on the mucous membrane of the larynx may be similar to that following exposure to mustard gas if sufficient HN_2 is delivered to these cells. Edema and vesiculation are produced (Klopp *et al.*, 1950a). Vesiculation of the pleura and localized areas of bullous emphysema of the lungs also have been described following exposure to mustard vapor. The lung alveoli react in the same manner as mucous membrane. Interstitial edema of the alveoli is produced and rapidly interferes with gaseous exchange; respiratory distress results. If a sufficiently large

amount of mustard vapor has been inhaled, there is an outpouring of fluid into the alveoli which produces severe coughing and further inhibits gaseous exchange. During the intravenous administration of HN_2, the capillary bed of the lung is the first to be traversed by the drug, which must therefore reach it in a somewhat greater concentration than is later delivered to the entire peripheral vascular network. No evidence is available to indicate that any effect similar to that produced by inhalation has ever been seen following intravenous administration. Minimal intracellular lung changes have been noted in small animals following intravenous administration (Skipper *et al.*, 1951), and massive necrosis of one lung has been produced in the dog by injection of a large dose of HN_2 into one pulmonary artery. The relatively increased effectiveness of intravenously administered HN_2 on lung cancer is still best explained as due to the delivery to the primary tumor of an increased amount of HN_2. This is the only clinical observation which suggests that the lung tissue removes any significantly increased amount of intravenously administered HN_2.

No information is available on the effect of the administration of HN_2 into either or both bronchial arteries.

VI. Gastrointestinal Tract

Reaction of the mucous membranes of the oral cavity to both mustard gas and to local intra-arterial administration of HN_2 has been described in the section on the respiratory tract. Early observations on experimental animals described unusual salivation following exposure to mustard gas (Warthin and Weller, 1918–19). Following the injection of HN_2 into the external carotid artery the salivary glands within the treated region became enlarged, tense and tender, and secreted increased amounts of saliva. This was intermittent at first, but became continuous after a week or more of therapy by the intra-arterial route. The effect on salivary gland secretion is attributed at least in part to the parasympathomimetic action of the HN_2, since the intermittent secretion can be prevented by administration of atropine. However, the later continuous phase is completely resistant to atropine therapy and, hence, is probably due to intracellular action of the drug. Following discontinuance of HN_2 therapy, the excessive salivation subsides as does the palpable enlargement of the glands. These effects must again be related to the concentration of drug delivered to the site of action because such changes never follow intravenous therapy or the intra-arterial injection of small amounts of HN_2.

Administration of a sufficient amount of HN_2 by any route is invariably followed by signs and symptoms of gastrointestinal irritation,

namely, nausea, vomiting, and diarrhea. Symptoms which followed the accidental exposure of combat personnel to sulfur mustard were attributed to the local irritant action of ingested mustard on the gastrointestinal tract mucosa. Subsequent observations on intravascularly treated patients suggest that these symptoms are produced, at least in part, by the action of HN_2 on nerve tissue, the peripheral parasympathetic nerves, or both. The administration of HN_2 into the lower aorta often produces less severe gastrointestinal tract symptoms than does intravenous administration. Injection of small doses into the internal carotid artery produces almost none (Klopp *et al.*, 1950a). Peripheral action appears to be most important. However, no information is available on the symptoms produced by the injection of HN_2 into the vertebral arteries which might supply a center sensitive to HN_2, the stimulation of which produces the gastrointestinal symptoms.

Local application of mustard to the gastric mucosa by swallowing produced inflammation, as noted in the Bari harbor casualties. The degree of inflammation would appear partially dependent on the concentration of the mustard delivered to the cells. No consistent evidence of irritation of gastric mucosa has been noted in cases of intravenously treated HN_2 patients, while the intra-arterial (celiac) administration of repeated small amounts of HN_2 in the dog produced inflammation and hyperemia in the stomach. Inconstant ulceration has been noted at autopsy in the stomach and colon following exposure to mustard gas (Alexander, 1947; Boxwell, 1919). Observations of the effect of HN_2 on gastric acidity are not available. In the presence of a high degree of acidity gastric irritation could be produced by this parasymphathomimetic drug. This could increase the secretory response to vagal stimulation.

The epithelial cells of the mucosa of the small intestine of the rat are quite sensitive to the action of HN_2. Microscopic changes are produced in them even when the drug has been given intravenously and intraperitoneally (Karnofsky *et al.*, 1948b). The first changes occur in the basilar glandular cells and are intranuclear. They consist of a clumping of nuclear chromatin and flattening of the cell shape. Finally, there is dilatation of the basilar portion of the gland itself. The distribution of changes is best explained as an effect on intranuclear components of the most rapidly dividing cells of the epithelium. These basilar cells have a rate of division comparable to that of the cells of blood and lymph-forming organs. The same explanation of the unexpected effect of HN_2 on nasopharyngeal cancer might apply to the gastrointestinal tract, i.e., the presence of an extremely rich lymphatic system in submucosal tissue which would collect the drug and expose the cells to an increased concentration.

Similar histologic changes are demonstrable in epithelial cells of

the glands of the colon of the rat following intra-arterial administration (Berry and Klopp). The same changes, but more minimal in degree, are seen following intravenous administration but then the effect is most marked in the ileum and progressively decreases in intensity down the gastrointestinal tract (Karnofsky *et al.*, 1948b). That the severity of these cytologic changes is due in part to the amount of HN_2 delivered is demonstrated by the increased effects noted following the intra-arterial as compared to intravenous injection in rats (Berry and Klopp), dogs (Barberio *et al.*, 1951) and at least one clinical case (Wintrobe *et al.*, 1947).

VII. Liver and Pancreas

Routine tests show no evidence of impairment of liver function by a standard course of HN_2 therapy (Alpert *et al.*, 1950; Goodman *et al.*, 1947; Jacobson *et al.*, 1946). Patients with evidence of previous damage to the liver have been treated with repeated courses of the drug and have shown no untoward effects as measured by routine function tests (Jacobson *et al.*, 1946). On the other hand, patients with extensive hepatic involvement plus ascites have, on the whole, responded poorly to HN_2 (Dameshek *et al.*, 1949). However, the response is variable. Two patients with clinically demonstrable hepatic enlargement associated with jaundice improved following HN_2 therapy (Dameshek, 1949).

Evidence has been presented to indicate that the liver cells play a significant role in detoxifying HN_2. There is no conclusive evidence that this process harms the liver cell. It has been pointed out by Friedenwald and Buschke (1948) that while some tissues with many dividing cells, such as bone marrow and intestinal mucosa, are very sensitive to mustard, a tissue like liver, with active cell division stimulated by partial hepatectomy, is comparatively insensitive. Another apparently unique response of liver tissue to HN_2 is demonstrated by an increase in citric acid synthesis following administration of this agent. Citrate formation is depressed in thymus and spleen. These observations were made on animals (Du Bois and Cochran, 1952) and resemble those following x-radiation. When the HN_2 is given in a single large dose or in repeated doses directly into the hepatic artery in human patients, no harmful effects can be detected by routine liver function studies. Also, when the administration of HN_2 is combined with that of intravascularly administered large single and total doses of glycine buffered aureomycin hydrochloride, there is no evidence that the HN_2 increases the degree of liver dysfunction which is produced by the large doses of intravascularly administered aureomycin (Bateman *et al.*, 1953).

Following the administration of lethal doses of mustard vapor to the

dog, hyperglycemia was produced, followed by profound hypoglycemia just before death; administration of sublethal amounts had no effect (Dziemian, 1946). A limited hypoglycemic action of HN_2 has been demonstrated in dogs; hepatic glycogen was difficult to evaluate in the sacrificed animals because, although generally lower than in control animals, the range in both groups was wide (Giordano and Bussinello, 1951). Observations regarding the effect of HN_2 in human patients are contradictory. Lowering of blood sugar in nondiabetic (Giordano and Rovasio, 1951; Green) and in diabetic individuals (Giordano and Rovasio, 1951) has been reported. No significant change in blood glucose level of nondiabetic individuals (Bateman *et al.*, 1951) and no influence on blood glucose level or urinary glucose excretion in diabetic patients (Saunders and Green, 1952) following HN_2 administration was observed by other workers. A hypoglycemic action by HN_2 is probably indirect and no doubt is related to amount and duration of therapy.

VIII. Kidneys

Standard courses of HN_2 have no demonstrable effect on normal kidney structures and renal function as determined by routine studies and tests. An increased urinary output following intravenous injection of HN_2 has been observed in rats. These animals excreted a test dose of water more rapidly than did control rats. However, no decrease in circulating antidiuretic substance such as follows whole body x-radiation could be demonstrated and different mechanisms are assumed to be involved (Edelmann *et al.*, 1952). The intra-aortic suprarenal injection of a single large dose of HN_2 produced microscopic changes in the glomeruli and proximal convoluted tubules of rats (Berry and Klopp). Similar intra-aortic injections on a few human patients have produced measurable depression of renal function, the decreases being noted in rate of glomerular filtration and renal blood flow (Kleh).

In a series of patients treated with large doses of HN_2 by the intra-arterial route, repeated determinations of the nonprotein nitrogen content of blood demonstrated the development of mild azotemia (Bateman *et al.*, 1951). How much of this can be attributed to influences on kidney function is, at present, not known. Decreases in nonprotein nitrogen of blood have also been noted, but could always be attributed to a decrease in degree of obstruction of the urinary tract resulting from a diminution in size of an obstructing tumor mass.

HN_2 has been used for the treatment of human glomerular nephritis and has produced in some cases a return of glomerular function toward normal (Boyd and Commons, 1952; Chasis *et al.*, 1950). Improvement could be attributed as much to an effect on the disease process as to a direct action on the kidney function.

The administration of a single massive dose of HN_2 into the renal artery of the dog has produced glomerular and vascular renal changes which are quite similar to those noted in essential hypertension. It has also produced moderate cytotoxic changes in the proximal convoluted tubules (Ayres). No immediate blood pressure change could be correlated with the injection of HN_2 into the renal artery of the dog (Ayres). It is interesting to note that hypertension was observed in military casualties at Bari harbor for a short time after exposure to and during recovery from toxic effects of the mustard (Alexander, 1947).

IX. Genital Tract

As ordinarily administered by the intravenous route, no demonstrable changes are produced in any organ of the genital tract with the possible exception of the testicles. Even here the presence of cytotoxic changes are not constant (Spitz, 1948).

As spermatic cells are regarded as extremely sensitive to the effects of irradiation and exposure to other agents which act primarily on rapidly dividing cells, this lack of a clear-cut demonstrable effect on spermatogenic elements is unexpected. A possible explanation is that the concentration and the amount of HN_2 delivered to the testicle is relatively small because of a small arterial blood supply and limited number of afferent lymphatic channels.

When high concentrations and large total doses of HN_2 are delivered to the cells of other organs of the genital tract, an effect is noted in some. Following intra-aortic injection, microscopic changes similar to those seen in the rectal epithelium have been observed in the glandular structures of the endometrium of the dog (Barberio *et al.*, 1951). No similar observations have been made on human patients. However, during the period of intra-aortic administration of HN_2, the number of cornified cells demonstrable in vaginal smears is increased (Cromer *et al.*, 1952). This is probably a direct action on the epithelium of the cervix and not an indirect hormonal effect. No significant change in menstrual function has been reported during or following HN_2 therapy.

X. Central Nervous System

Intravenous administration of usual amounts of HN_2 has no apparent effect on either the peripheral or central nervous system. When larger concentrations are delivered to a specific region, effects on nerve tissue and function have been noted. After injections into one external carotid artery, ipsilateral motor paralysis of the seventh and twelfth cranial nerve has been produced in some patients. In a few instances, when injections were given into both external carotid arteries, dysphagia was produced (Klopp *et al.*, 1950a). Although some of this dysphagia could

be attributed to neuromuscular dysfunction, it was undoubtedly aggravated by the accompanying edema and mucositis. Injection into the main artery of an extremity of a patient has never produced paralysis, but has been followed by a transient muscular weakness.

The intra-arterial administration of HN_2 in the therapy of cancer patients resulted in relief of pain whenever the pain was due directly to the malignant tumor (Klopp *et al.*, 1950a,b; Schwarz *et al.*, 1952). This pain relief has been attributed to the regression of the cancer. No sensory loss has been noted within the treated region during or following therapy. Partial or complete loss of motor function has been seen during therapy in some cases. Anatomical nerve changes have been found on histologic study of nerves obtained at autopsy from treated regions. This evidence of change in peripheral nerves suggests the possibility that pain relief may, at least in part, be due to the direct action of HN_2 on the sensory nerve fibers.

An effect of HN_2 on the function of nerve cells might have been expected as HN_2 has, in physiological concentrations, an inhibitory action on certain oxidases and esterases (Barron *et al.*, 1948; Karnofsky, 1950), including choline esterase. Once produced, this inhibition of choline esterase is difficult to reverse and the reaction is not directly related to the concentration of HN_2 employed (Barron *et al.*, 1948). This observation, together with the known ability of HN_2 to inhibit partially other enzymes (Barron *et al.*, 1948a) would supply a plausible physiological explanation for the effects noted on motor function, namely, the muscular weakness which sometimes follows regional intra-arterial therapy. The site of action is presumably the myoneural junction. Intra-arterial injections given over a prolonged period of time might have an additional effect, that of anatomical nerve damage which could produce the posterior limb paralysis seen in the dog (Barberio *et al.*, 1951), rat (Berry and Klopp), and the facial nerve paralysis noted in a few humans.

The parasympathomimetic action of HN_2 has previously been discussed in relation to salivary glands. Similar action has been noted on the iris following intra-arterial therapy and on blood vessels of regional areas, as indicated by the increase in skin temperature (Klopp *et al.*, 1950). Such action, like that on motor nerves, is attributed to an effect on the acetylcholine choline-esterase system, the latter being inactivated specifically by HN_2 permits the former to accumulate. This would produce the parasympathomimetic overactivity which has been noted.

Serial electroencephalographic tracings taken on cats and monkeys following injection of HN_2 into the carotid artery showed an initial transient change. Days later, depending on the dose injected, asymmetry of the tracings developed, the injected side showing low voltage activity

as compared to the uninjected. Pathologic changes depended on the amount of HN_2 administered and the time interval. Early changes included ischemia and congestion; later, edema, petechial ring hemorrhage, patchy areas of thrombi, varicose degenerated vessels, perivascular phagocytes, chronic neuronal degeneration, areas of demyelinization, and increase of cortical glia were observed (French *et al.*, 1952).

In 1919 Moorehead reported the presence of drowsiness, lethargy, headache, hypotension, and rapid pulse in victims of severe mustard gas poisoning. In the report of the Bari harbor casualties, Alexander (1947) noted the presence of apathy and severe hypotension and the absence of a perceptible pulse. Of these latter cases, some who subsequently recovered had a temporary elevation of systolic blood pressure during the period of recovery. These central nervous system manifestations were considered to be due to the absorption of large amounts of mustard gas. This concept is strengthened by observations on the initial group of cancer patients treated by the repeated injection of HN_2 into arteries. Among these cases were many patients who received a large total dose and, of these, hypotension and marked weakness developed in a few. These patients were mentally alert but required a strong stimulus to elicit a response. The weakness was so severe that talking became an effort. While the blood pressure was decreased and the pulse was rapid, true shock was not present as extremities were warm and dry. No marked changes in components of the blood were noted. The clinical picture was attributed to a direct action of HN_2 on the central and peripheral nervous system (Klopp *et al.*, 1950). These observations were almost identical to those noted in victims of the Bari harbor incident where the shocklike state was described as consisting of severe hypotension, imperceptible pulse, apathy without restlessness, anxiety, and distress, but with the presence of warm extremities. In such cases any therapy directed at treatment of shock, such as the use of external heat, fluids, morphine, and even of blood, was totally ineffective (Alexander, 1947; Klopp *et al.*, 1950).

Toxic psychosis has been reported to follow intravenous administration of HN_2 in a 25-year-old man with Hodgkin's disease (Roswit and Pisetsky, 1952). The symptoms included restlessness, euphoria, and confusion. There was complete clearing in three weeks and no recurrence during subsequent treatment.

XI. Endocrine Glands

Administration of HN_2 has produced no specific demonstrable anatomical change in endocrine glands. Following administration of toxic doses of HN_2, the adrenal gland shows changes consistent with the

alarm reaction (Karnofsky *et al.*, 1948b; Ludewig and Chanutin, 1946). Following intra-aortic supra-adrenal injection of a single large dose in the rat, focal areas of necrosis were observed (Berry and Klopp). The appearance of hirsutism in females with Hodgkin's disease has been noted after repeated courses of HN_2 (Ben-Asher, 1949). Certain other changes are similar to those associated with the alarm reaction, namely, immediate decrease in peripheral eosinophil and lymphocyte counts, early transient granulocytosis, evidence of destruction of lymphoid tissue (Gilman, 1947), and electrolyte losses as demonstrated in the dog (Philips *et al.*, 1948). This ACTH-like effect may, to a large degree, be attributable to the genuine alarm reaction, but might also in part be a direct action of the HN_2 on the adrenals. This has been suggested by the increased lymphocyte response noted in dogs receiving intra-aortic suprarenal injections as compared to animals receiving intravenously administered HN_2. Such action might also account for the relatively greater beneficial effect of HN_2 on Hodgkin's disease as compared to other malignant tumors, and on such disease as lupus erythematosus (Osborne *et al.*, 1947), rheumatoid arthritis (Diaz *et al.*, 1951), and nephritis (Chasis *et al.*, 1950).

Other suggestive similarities between the effect of administration of HN_2 and ACTH have been described. Not infrequently, following a course of HN_2, patient's with Hodgkin's disease have a dramatic but transient return to a feeling of well-being, a disappearance of fever, a marked increase in appetite, and gain in weight. Pruritis may disappear. Some patients treated for a prolonged period show a consistent decrease in serum albumin and an increase in globulin following HN_2 therapy (Bateman *et al.*, 1951). Furthermore, HN_2 has an effect on immunological response not dissimilar to that produced by ACTH. In animals, a course of HN_2 suppresses antibody response to injection of typhoid vaccine (Spurr, 1947). HN_2 inhibits antibody production, arthus reaction and vascular lesions in rabbits sensitized to horse serum (Dammin and Bukantz, 1949). Its administration inhibits the Schwartzman reaction (Schlang, 1950). This latter action is complete in rabbits but only when HN_2 is administered three to four days prior to the injection of the reacting factor (Becker, 1948). Insulin-resistant diabetes precipitated by cortisone has been reversed by HN_2 (Geller *et al.*, 1951).

XII. Metabolic Changes

Most balance studies during HN_2 administration have been done on animals. A few have been reported on humans. In the dog, increase in urinary output has been noted (Flury and Wieland, 1921; Philips *et al.*, 1948). In rats the mean rate of proteinuria induced by the intraperitoneal

administration of human serum albumin was increased in HN_2-intoxicated animals (Lippman and Ureen, 1951). In 2 patients with advanced cancer who received intra-arterially administered HN_2 there was a significant increase in the negative nitrogen balance during the first 24 hours. This was not maintained during the subsequent days of therapy (Jacobson *et al.*, 1948). Increased cellular catabolism is considered to account for the increased excretion of nitrogen.

Marked loss of sodium, potassium, and chlorides was observed in HN_2-treated animals (Philips *et al.*, 1948) and depression in serum sodium and elevation of serum potassium were seen in treated human patients. Balance studies, however, demonstrated no significant increase in output of sodium in the latter.

An effect on resistance to infection has been encountered. Enhanced bacterial growth of hemolytic streptococci occurs consistently in the serum of HN_2-intoxicated rabbits beginning 6 to 8 hours after administration (Karnofsky *et al.*, 1948). In our initial intra-arterially treated patients, the incidence of local and systemic infection was higher than had been anticipated, and at least three instances of bacterial endocarditis occurred during therapy. This complication could not be correlated with the degree of leukopenia. Following the concomitant intravascular administration of antibiotics, this type of infection was no longer seen.

XIII. Methods for Counteracting Toxic Effects

Therapeutic application of HN_2 has been limited by the poisonous effect of this agent on host as well as tumor. Some of these effects, such as nausea and vomiting, have chiefly a nuisance value, assuming a serious character only in special cases. However, severe nausea and vomiting have a psychological effect that is particularly unfortunate when therapy needs to be repeated at frequent intervals. Systemic effects such as depression of bone marrow, disturbance of electrolyte balance and interference with immunological responses become absolute limiting factors in the amount of drug that can be administered.

When NH_2 is injected intravenously, all functioning bone marrow is about equally affected. It has been demonstrated by simultaneous marrow studies from sternum, ribs, and iliac crest that HN_2 administered into the lower abdominal aorta has an earlier and more profound effect on iliac marrow than on sternal and rib marrow (Bateman *et al.*, 1951). Clamping of the abdominal aorta and vena cava in rats for 20 to 60 minutes after the injection of mustard gas resulted in appreciably less severe damage to femoral marrow than to humeral marrow (Needham *et al.*, 1947). However, the protection can be overcome when a dose of sufficient size is administered (Karnofsky *et al.*, 1948b; Needham *et al.*,

1947). Blood pressure cuffs have been applied to the thighs and inflated above systolic pressure for 5 to 10 minutes as HN_2 was administered to human patients, in the hope of protecting bone marrow (Karnofsky *et al.*, 1948a). This was unsuccessful. The difference in the results produced by this technic in animals and humans may be explained by the fact that marrow in the extremities of animals is hematopoietically functional; that of human beings is only potentially so except in the presence of severe anemia.

Various agents have been used in an attempt to counteract the nauseous action of HN_2. Barbiturates given prior to the injection are effective (Falloon and Gorham, 1948). Pyridoxine hydrochloride was given with questionable results when administered 30 minutes after HN_2 in order to allow time for fixation of the latter in tissue and to prevent any inhibition by the former (Bauer and Erf, 1950).

Inhibition of bone marrow respiration *in vitro* by HN_2 has been prevented by the addition of choline and of dimethoamino ethanol plus methionine (Barron *et al.*, 1948b). Choline administration has been employed during therapy but no clear protective action has been demonstrated.

Cysteine administered prior to injection of HN_2 reduced toxicity of the latter drug in animals (Brandt and Griffin, 1951; Griffin and Brandt, 1951; Weisberger and Heinle, 1950; Weisberger *et al.*, 1951b) and in human patients (Weisberger *et al.*, 1951a,b; Winship). Patients given cysteine prior to HN_2 had a fall in white blood cell count of approximately 62% as compared to 83% in those given HN_2 alone. There was no interference with the therapeutic activity of the latter.

A prolonged clotting time and decreased "heparin tolerance" has been described in rabbits and in human beings after therapeutic doses of HN_2 (Jacobson *et al.*, 1948). Protamine was used to correct this defect. In another series of patients only minimal prolongation of coagulation and bleeding times was observed. Even when platelet counts dropped to fairly low levels, no bleeding tendencies were noted (Bateman *et al.*, 1951).

Since cortisone stimulates granulopoiesis and erythropoiesis and causes retention of some electrolytes, its use as an adjuvant in HN_2 therapy seemed logical. A group of patients who were given large doses of HN_2 by the arterial route received daily intramuscular injections of cortisone. Bone marrow depression was delayed but unless cortisone was continued after completion of the course of HN_2, prolonged severe depression of marrow ensued. Electrolyte balance studies were not done; however, cortisone-treated patients lost less weight than those given HN_2 alone.

Large doses of aureomycin given concomitantly with HN_2 in intra-

arterially-treated patients not only reduced the incidence of infection but also maintained nutrition in some and showed a tendency to protect the bone marrow (Bateman *et al.*, 1953).

Other agents, such as pentose nucleotides, liver extract, and pteroylglutamic acid have been recommended (Kreiner and Bauer, 1951), but there is only the clinical impression that they are effective. In general, the mechanical methods and numerous chemical agents used to protect the host from systemic injury by HN_2 decrease in effectiveness as the dose of HN_2 is increased.

REFERENCES

Achard, C. 1919. *Bull. acad. méd. (Paris)* **81,** 135.

Adair, F. E., and Bagg, H. J. 1931. *Ann. Surg.* **93,** 190.

Alexander, S. F. 1947. *Military Surgeon* **101,** 1.

Algire, G. H., and Chalkley, H. W. 1945. *J. Natl. Cancer Inst.* **6,** 73–85.

Alpert, L. K., Greenspan, E. M., and Petersen, S. S 1950. *Ann. Internal Med.* **32,** 393–432.

Anslow, W. P., Jr., Karnofsky, D. A., Jager, B. V., and Smith, H. W. 1948. *J. Pharmacol. Exptl. Therap.* **93,** 1.

Apthomas, M. I. R., and Collumbine, H. 1947. *Lancet* **I,** 899.

Axelrod, D. J., and Hamilton, J. G. 1947. *Am. J. Pathol.* **23,** 389.

Ayres, W. W. Personal communication.

Barberio, J. R., Klopp, C. T., Ayres, W. W., and Gross, H. A. 1951. *Cancer* **4,** 1341.

Barron, E. S. Guzman, Bartlett, G. R., and Miller, Z. B. 1948a. *J. Exptl. Med.* **87,** 489–501.

Barron, E. S. Guzman, Bartlett, G. R., Miller, Z. B., Meyer, J., and Seegmiller, J. E. 1948b. *J. Exptl. Med.* **87,** 503.

Bateman, J. C., Barberio, J. R., Cromer, J. K., and Klopp, C. T. 1953. *J. Antibiot. Chemother.* **3,** 1–15.

Bateman, J. C., Klopp, C. T., and Cromer, J. K. 1951. *Blood* **6,** 26–38.

Bauer, R. D., and Erf, L. A. 1950. *Am. J. Med. Sci.* **219,** 16–26.

Becker, R. M. 1948. *Proc. Soc. Exptl. Biol. Med.* **69,** 247–250.

Ben-Asher, S. 1949. *Am. J. Med. Sci.* **217,** 162.

Berg, H. F. 1951. *Arch. Surg.* **63,** 545–553.

Berry, G. N., and Klopp, C. T. Intra-Arterial Nitrogen Mustard Toxicity Study in Rats. Submitted for publication.

Bierman, H. R., Byron, R. L., Jr., and Kelly, K. H. 1951a. *Cancer Research* **2,** 236.

Bierman, H. R., Byron, R. L., Jr., Miller, E. R., and Shimkin, M. B. 1950a. *Am. J. Med.* **8,** 535.

Bierman, H. R., Kelly, K. H., Byron, R. L., Dod, K. S., and Shimkin, M. B. 1951b. *J. Natl. Cancer Inst.* **11,** 891–905.

Bierman, H. R., Kelly, K. H., Dod, K. S., and Byron, R. L. 1951c. *J. Natl. Cancer Inst.* **2,** 877–884.

Bierman, H. R., Shimkin, M. B., Byron, R. L., Jr., and Miller, E. R. 1950b. *5th Intern. Cong. Cancer (Paris)*, pp. 16–22.

Block, M. H., Spurr, C. L., Jacobson, L. O., and Smith, T. R. 1948. *Am. J. Clin. Pathol.* **18,** 671.

Boyd, R. I., and Commons, R. R. 1952. *Am. J. Med.* **13,** 499.

Boxwell, W. 1919. *Irish J. Med. Sci.* **147,** 7–10.

Brandt, E. L., and Griffin, A. C. 1951. *Cancer* **4,** 1030–1035.
Cameron, G. R., and Courtice, F. C. 1948. *Quart. J. Exptl. Physiol.* **34,** 165–179.
Chasis, H., Goldring, W., and Baldwin, D. S. 1950. *J. Clin. Invest.* **29,** 804.
Cornman, Ivor. Personal communication.
Craver, L. F. 1948. *Bull. N.Y. Acad. Med.* **24,** 3.
Cromer, J. K., Bateman, J. C., Berry, G. N., Kennelly, J., Klopp, C. T., and Platt, L. I. 1952. *Am. J. Obst. Gynecol.* **63,** 538–548.
Dameshek, W., Weisfuse, L., and Stein, T. 1949. *Blood* **4,** 338–379.
Dammin, G. J., and Bukantz, S. C. 1949. *J. Am. Med. Assoc.* **139,** 358.
Diaz, J., Perianes, M., Garcia, L., and Leal, P. 1951. *J. Am. Med. Assoc.* **145,** 1365.
DuBois, K. P., and Cochran, K. W. 1952. *Federation Proc.* **11,** 341.
Dziemian, A. J. 1946. *Federation Proc.* **5,** 175.
Edelmann, A., Healey, R. B., and Mateyko, G. M. 1952. *Federation Proc.* **11,** 41.
Falloon, W. W., and Gorham, L. W. 1948. *N.Y. State J. Med.* **48,** 612.
Flury, F., and Wieland, H. 1921. *Z. ges. exptl. Med.* **13,** 367.
French, J. D., West, P. M., von Amerongen, F. K., and Magoun, H. W. 1952. *J. Neurosurg.* **9,** 378–389.
Friedenwald, J. S., Buschke, W., Scholz, R. O., and Moses, S. G. 1947. Approaches to Tumor Chemotherapy, p. 358. Am. Assoc. Advance. Sci., Washington, D.C.
Friedenwald, J. S., and Buschke, W. 1948. *Bull. Johns Hopkins Hosp.* **82,** 161–178.
Geller, W., LaDue, J. S., and Glass, G. G. J. 1951. *Arch. Internal Med.* **87,** 124.
Gilman, A. 1946. *Federation Proc.* **5,** 285.
Gilman, A. 1947. *Am. J. Med.* **2,** 209.
Giordano, G., and Bussinello, E. 1951. *Boll. Soc. ital. biol. sper.* **27,** 1243–1244.
Giordano, G., and Rovasio, P. 1951. *Boll. Soc. ital. biol. sper.* **27,** 1241–1243.
Goodman, L., Wintrobe, M. M., McLennan, M. T., Dameshek, W., Goodman, M. J., and Goodman, A. 1947. Approaches to Tumor Chemotherapy, p. 339. Am. Assoc. Advance. Sci., Washington, D.C.
Green, D. M. Personal communication.
Griffin, A. C., and Brandt, E. L. 1951. *Federation Proc.* **10,** No. 1, Part 1.
Guthrie, F. 1860. *J. Am. Chem. Soc.* **12,** 108.
Jacobson, L. O., Marks, E. K., Gaston, E., Allen, J. G., and Block, M. H. 1948. *J. Lab. Clin. Med.* **33,** 1566.
Jacobson, L. O., Spurr, C. L., Guzman Barron, E. S., Smith, T., Lushbaugh, C. C., and Dick, G. F. 1946. *J. Am. Med. Assoc.* **132,** 263.
Karnofsky, D. A. 1950. *Advances in Internal Med.* **IV.**
Karnofsky, D. A., Abelmann, W., Craver, L. F., and Burchenal, J. H. 1948a. *Cancer* **1,** 634.
Karnofsky, D. A., Graef, I., and Smith, H. W. 1948b. *Am. J. Pathol.* **24,** 275.
Kleh, J. Personal communication.
Klopp, C. T., Alford, T. C., Bateman, J. C., Berry, G. N., and Winship, T. 1950a. *Ann. Surg.* **132,** 811–831.
Klopp, C. T., Bateman, J. C., Berry, G. N., Alford, T. C., and Winship, T. 1950b. *Cancer Research* **10,** 229.
Kreiner, W. M., and Bauer, L. 1951. *J. Am. Med. Assoc.* **143,** 188.
Krumbhaar, E. B. 1919a. *J. Am. Med. Assoc.* **72,** 39.
Krumbhaar, E. B. 1919b. *J. Am. Med. Assoc.* **73,** 715.
Krumbhaar, E. B., and Krumbhaar, H. D. 1919–1920. *J. Med. Research* **40,** 497–508.
Lippman, R. W., and Ureen, H. J. 1951. *Federation Proc.* **10,** No. 1, Part 1.
Ludewig, S., and Chanutin, A. 1946. *Endocrinology* **38,** 376.

Lynch, J. L., Ware, P. F., and Gaensler, E. A. 1950. *Surgery* **27,** 368–385.
Meyer, V. 1887. *Ber. deut. chem. Ges.* **20,** 1729.
Moorehead, T. G. 1919. *Irish J. Med. Sci.* **147,** 1–7.
Needham, D. M., Cohen, J. A., and Barrett, A. M. 1947. *Biochem. J.* **41,** 631.
Osborne, E. D., Jordan, J. W., Hoak, F. C., and Pschierer, F. J. 1947. *J. Am. Med. Assoc.* **135,** 1123.
Philips, F. S., Gilman, A., Koelle, E. S., McNamara, B. P., and Allen, R. P. 1948. *Am. J. Physiol.* **155,** 295.
Roswit, B., and Pisetsky, J. E. 1952. *J. Nervous Mental Disease* **115,** 356–359.
Saunders, J., and Green, D. M. 1952. *Federation Proc.* **11,** 394.
Schlang, H. A. 1950. *Proc. Soc. Exptl. Biol. Med.* **74,** 749–751.
Schwarz, H., Castanares, S., and McAleese, G. 1952. *Arch. Surg.* **64,** 286–291.
Seligman, A. M., Friedman, O. M., and Rutenburg, A. M. 1950. *Cancer* **3,** 342–347.
Skipper, H. E., Bennett, L. L., and Langham, W. H. 1951. *Cancer* **4,** 1025–1047.
Smith, T. R., Jacobson, L. O., Spurr, C. L., Allen, J. G., and Block, M. H. 1948. *Science* **107,** 474.
Spitz, S. 1948. *Cancer* **1,** 383.
Spurr, C. L. 1947. *Proc. Soc. Exptl. Biol. Med.* **64,** 259–261.
Spurr, C. L., Jacobson, L. O., Smith, T. R., and Barron, E. S. Guzman 1947. Approaches to Tumor Chemotherapy, p. 306. Am. Assoc. Advance. Sci., Washington, D.C.
Spurr, C. L., Smith, T. R., and Jacobson, L. O. 1948. *Radiology* **50,** 387.
Taffel, M. 1947. *Yale J. Biol. Med.* **19,** 971.
Ward, K. 1935. *J. Am. Chem. Soc.* **57,** 914.
Warthin, A. S., and Weller, C. V. 1919. The Medical Aspects of Mustard Gas Poisoning. St. Louis, C. V. Mosby Co.
Warthin, A. S., and Weller, C. V. 1918–1919. *J. Lab. Clin. Med.* **4,** 265.
Wawro, N. W. 1948. *Connecticut State Med. J.* **12,** 625–631.
Weisberger, A. S., and Heinle, R. W. 1950. *J. Lab. Clin. Med.* **36,** 872–876.
Weisberger, A. S., Heinle, R. W., and Levine, B. 1951a. *Proc. Am. Fed. Clin. Research.*
Weisberger, A. S., Heinle, R. W., and Levine, B. 1951b. *J. Clin. Invest.* **30,** 681.
Winship, T. Personal communication.
Wintrobe, M. M., Huguley, C. M., Jr., McLennan, M. T., and Lima, L. P. de C. 1947. *Ann. Internal Med.* **27,** 529.
Wintrobe, M. M., and Huguley, C. M., Jr. 1948. *Cancer* **1,** 357.

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Genetic Studies in Experimental Cancer

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CONTENTS

I. Introduction

An inherited disease is one in which the condition is determined, wholly or in part, by the genetic constitution with which the organism started its development. The genetic constitution of an organism is made up of discrete, stable units, the genes, which are carried in the chromo-

somes in linear order. Susceptibility to cancer is inherited, as the wealth of evidence will show; the concept of the problem, at present, is not in showing that it is gene controlled, its causal genesis, but in elucidating the mechanisms leading to its appearance, its formal genesis. The determination that some forms of cancer in experimental animals are gene controlled is not the end of the story; it is merely the beginning.

An attempt is made here to present contributions to our present knowledge of the etiology of cancer as revealed in genetic studies employing inbred strains of mice. Such critical studies, in addition to establishing the role of the gene in the etiology of cancer, have uncovered some striking and still other subtle, nongenetic influences, such as the mammary-tumor milk agent, the maternal resistance factor in leukemia, and maternal influence in expressivity of fibrosarcomas. These have been given special emphasis. There is no intention of making the review complete, although findings are presented in their proper historical perspective. Recent work is emphasized and indications of future experimentation in the field are given.

In addition, a section has been included on immunogenetics with special emphasis on the genic control of tumor specificity. Much work has been done in the cancer field: in chemotherapy, tumor immunity, virus etiology, and the like without due regard to the fitness of the genetic system employed. It is hoped that an understanding of the more recent work on histocompatibility markers, the development of isogenic resistance strains of mice, etc., will form the basis for more critically designed experiments.

II. Genetics of Spontaneous and Induced Neoplasms

1. Mammary Cancer

The typical mammary gland tumors of the mouse are epithelial in origin, constituting for the most part adenocarcinomas and carcinomas (Dunn, 1945). A rigid classification is difficult because of the histologic variation seen in different areas of the same tumor. A difference in certain histologic features is apparent when inbred strains with and without the mammary tumor milk agent are compared (Heston *et al.*, 1950; Kirschbaum, 1949). Especially significant in mice without the agent, the C3Hb line, is the increase in tumors of the adenoacanthomatous type. This histologic type has been reported also by Kirschbaum *et al.* (1946) in DBA agent-free mice painted with a carcinogenic hydrocarbon.

Inbred strains of mice differ strikingly and consistently in the incidence of mammary tumors (Table I). Variations in incidence reported from different laboratories result from the development of sublines differ-

ing genetically and differences in nongenetic influences which affect the general mortality of the strains.

TABLE I
Incidence of Mammary Cancer in Various Inbred Strains of Mice*

Strain	Breeding Females			Virgin Females		
	No.	Incidence	Mean Tumor Age (months)	No.	Incidence	Mean Age (months)
C3H	1588	91.4	8.6	350	97.4	10.4
	200	78.0	10.7			
	419	97.0	7.8	46	100.0	10.6
A	972	95.1	8.9	223	4.9	18.5
	730	72.0	12.1			
	348	73.0	9.8			
DBA	1363	51.0	10.5	207	11.5	15.8
	80	76.3	12.7	197	72.1	13.4
Marsh	1350	76.4	13.7	74	68.9	13.5
RIII	140	73.6	12.2	94	52.1	16.4
BALB/c	178	1.1	24–29			
C57BL	240	0.0	—			
	568	0.5	18–29			
CBA	125	13.5	21.0			

* Data obtained from various sources. See especially Heston (1945) and Grüneberg (1952a).

A. *Genetic Studies.* Much of the early work done on the genetics of mammary cancer is invalidated because of the use of heterogeneous material. Pedigree studies showing cancerous mice and those which died from other causes were interpreted as showing clearly the simple Mendelian nature of inheritance. Since there appears to be no maximum age for the development of cancer and since in genetically heterogeneous material the probability of developing cancer is variable from animal to animal and unknown for any one individual, the classification of an animal as noncancerous reveals nothing about its potentialities (Grüneberg, 1952a).

In an inbred strain of mice the individuals are alike genetically except for mutations of recent origin which have not been established or elimi-

nated. An inbred mouse is not judged by the fact that it is cancerous or noncancerous, but it is judged by and its behavior is predicted from the inbred strain as a whole. This is much unlike interpreting pedigrees *ex post* in heterogeneous material, in which each individual differs genetically from another.

Clearly, the use of tumor incidences, in comparing inbred strains is not an ideal method. The tumor rate is influenced by the general mortality of the strain, and if many mice die noncancerous, fewer are left to develop cancer. An actuarial function such as that developed by Spicer

TABLE II

Maternal Influence in Mammary Cancer

Incidence in virgin females of DBA and C57BL strains and their reciprocal F_1 and F_2 hybrids*

Strain and Cross	Total No.	No. Mammary Cancer	Per Cent Mammary Cancer
DBA (D)	297	151	50.8
C57BL (B)	240	0	0
DBF_1	113	45	39.8
BDF_1	379	23	6.1
DBF_2	664	236	35.5
BDF_2	607	41	6.0

* Data of Murray and Little (1935).

(1947) would take into account these differences. It is doubtful that such a function is useful, however, in dealing with most strains where either a high or a low incidence is encountered. The extreme variation seen in the mammary tumor incidence of the DBA strain probably is the result of differences in general mortality in different laboratories.

Studies on the etiology of mammary cancer in mice began more than 40 years ago and pertinent reviews on the subject may be found by Bittner (1942b, 1945), Heston (1942a, 1945, 1948, 1951), Little (1947), National Cancer Institute Staff (1945), and Grüneberg (1952a).

In 1933 the staff of the Jackson Memorial Laboratory and in 1934 Korteweg published results of experiments which stimulated an enormous amount of experimental work on the etiology of mammary cancer in the mouse. Reciprocal crosses made between high- and low-mammary-cancer strains revealed a striking feature of the F_1 hybrid animals. The tumor incidence was found to be high where the mother was from the high-tumor strain and significantly lower where the mother was from the low-tumor strain. Since the genetic constitution of F_1 mice is identical both as regards autosomes and sex chromosomes, it was obvious that the

mammary cancer incidence was strikingly influenced by nongenetic factors, that is something not carried by the chromosomes. The difference was found to persist in reciprocal F_2 generations and later found by Murray and Little (1935) to persist in the backcross generation. This fact proved that the extrachromosomal effect was not a temporary prenatal influence but something handed down in the maternal line. Some typical results are given in Table II. Thus, it was clear that (*a*) something transmitted by way of the cytoplasm of the egg, (*b*) genetic or nongenetic

TABLE III

Influence of Mammary-Tumor Milk Agent on Incidence of Mammary Cancer

Strain of Mice		Strain of Foster Mother		Change in Tumor Incidence of Fostered Mice
C57BL	(low)	A	(high)	0.5 → 11.3
A	(high)	C57BL	(low)	83.6 → 6.4
C3H	(high)	C57BL	(low)	97.0 → 4.0*
BALB/c	(low)	C3H	(high)	1.4 → 81.9†
C57BL	(low)	C3H	(high)	0 → 2.3‡
DBA	(high)	$C3H_b$	(low)	43 → 0.6

* In virgin females; incidence in breeding females, 38% (Heston *et al.*, 1950). In contrast, Bittner's C3H strain, fostered on C57BL, and continued by sib-matings for 30 generations gave less than 1% tumors in breeding females.

† F_{11}-F_{23} generations following foster nursing (Andervont, 1945a).

‡ Incidence through 4 generations. Although evidence of the milk agent was found in the original fostered animals it apparently was not propagated in later generations (Andervont, 1945c). A much higher incidence of mammary tumors has been recorded by other investigators and in Bittner's subline of C57BL it would appear that the milk agent is propagated through successive generations (Bittner 1940, 1948).

prenatal influences, or (*c*) transmission of something through the mother's milk might account for the observed facts.

Through the efforts of Bittner (1936, 1937a,b,c) it was found that the extrachromosomal influence was in fact transmitted by mothers to their offspring by the milk. When mice of a high-mammary-cancer strain (A) were suckled by foster mothers of a low-cancer strain (CBA), the foster-nursed animals showed a strikingly lowered incidence of mammary tumors. This work has been extended and confirmed in numerous reports (see Andervont, 1945b). Table III gives some typical results showing also the converse relationship, the development of a high-mammary-tumor susceptibility following fostering of low-cancer groups by high-cancer females.

Fekete and Little (1942) have investigated possible intra-uterine influences by reciprocal transplantation of fertilized ova between high- and low-mammary-cancer strains. Although their results indicate an

additional extrachromosomal influence, corroboration of this work, in the absence of the complicating mammary-tumor milk agent, is needed.

There is no evidence available concerning transmission of the extrachromosomal influence by cytoplasm of the ovum as originally suggested by Korteweg (1934). It should be pointed out, however, that the standard test used to determine the presence of the milk agent, induction of tumors in susceptible test animals, may not be a crucial test of infection (Andervont, 1950; Andervont and Dunn, 1950a).

As proposed by Bittner (1939b), the development of mammary cancer in mice in general depends upon the action of three factors: (1) "the inherited susceptibility," or more appropriately the genotype of the individual, (2) hormonal stimulation, and (3) the mammary-tumor milk agent. An attempt will be made here to analyze the action and interaction of all three influences.

B. *The Mammary-Tumor Milk Agent.* The discovery of a mammary-cancer-inducing agent in the milk of certain strains of mice, Bittner (1936), was followed by a number of studies on the properties of this agent. The agent is widely distributed throughout the body of the mouse, being found in the spleen, thymus, lactating mammary glands, whole blood of both males and females, blood cells and serum, and in seminal vesicles (see review by Dmochowski and Passey, 1952). The properties of the agent in extracts of various normal and cancerous tissues were found to be similar to those of viruses. The agent remains active after lyophilization and after filtration through Seitz and Berkefeld filters (Bittner, 1942a), after desiccation (Dmochowski, 1944), and after treatment with glycerin (Bittner, 1942a). In extracts, the agent remains stable over a wide pH range from 5.0–10.2 and is not inactivated by acetone or petroleum ether (Barnum *et al.*, 1944). Heating of tissue extracts for one hour at 60°C. destroys its activity (Andervont and Bryan, 1944; Barnum *et al.*, 1944).

The agent remains active in dilutions of 1:1,000,000 (Barnum *et al.*, 1946). It is propagated only in the presence of living cells, and when introduced into suitable test mice, it produces mammary tumors and is propagated in successive generations of these mice (Andervont, 1945b).

Under certain experimental conditions the mammary tumor milk agent has been found to be antigenic. Andervont and Bryan (1944) described neutralization of the milk agent both *in vivo* and *in vitro* in material obtained by ultracentrifugation of extracts of mammary tumors. The rabbit was used to prepare immune sera. Green *et al.* (1946), and Green and Bittner (1946) showed similar neutralization in ultracentrifugal material of mammary cancer extracts. In none of these experiments was it possible to decide whether the neutralizing effects resulted from

antibodies to the milk agent or the tumor tissue. Imagawa *et al.* (1948) demonstrated that rabbit sera against normal and cancerous tissues with the milk agent gave a high titer in precipitin tests. Control experiments using mammary-cancer tissue without the milk agent were not reported. Bennison (1948) gave preliminary results suggesting complement-fixing antibodies to the milk agent, but more recent work of Dmochowski and Passey (1952) showed that the antibody in complement-fixation tests is not specific for the milk agent and furthermore agree with the results of Gorer and Law (1949) and Imagawa *et al.* (1951) on the absence of neutralizing and cytotoxic antibodies in sera of mice.

The propagation of the milk agent is not fully understood. It is not known for example whether the agent reproduces itself or whether susceptible mice are stimulated to produce more of the agent following administration of a small initial dose. One strain of mouse, the C57BL strain, has been shown by Andervont (1945c) not to transmit the agent with any degree of regularity to its young and the agent gradually disappears, as determined by the standard biologic test, in subsequent generations. It is unlikely that neutralization of the agent occurs (Gorer and Law, 1949), but that there is a negligible propagation (Andervont, 1952) that has a genetic basis. This will be discussed later.

More recently Green *et al.* (1946) and Andervont (1949) have reported diminution of the milk agent in successive generations of offspring in susceptible mice of the C3H and BALB/c strains following introduction of a small amount of agent. On the other hand, Bittner (1941) has shown the development of a high-cancerous line, in A strain mice, previously foster-nursed, the ancestors of which failed to show mammary tumors for 7 successive generations. The original litter from which the line developed remained with their A mother for a short time and in all probability obtained some milk. Thus, a satisfactory explanation is increased rate of propagation of the agent although Bittner has suggested a change from an "inactive" to an "active" influence or *de novo* appearance of the agent. The suggestion of a "pre-viral or inactive viral" stage, therefore, followed by activation to the true cancer-producing virus" (Graff *et al.*, 1952) would seem premature in the light of our present knowledge.

Although there is a long latent period of mammary cancer development in mice, certain infectious viruses behave in a similar manner, especially the so-called scrapie virus in sheep (Greig, 1940).

The probable transmission of the mammary-tumor milk agent through the sperm of mice (Andervont and Dunn, 1948; Mühlbock, 1950) resembles similar transmission of the scrapie virus and the virus of fowl paralysis (Blackmore, 1934).

The difficult task of determining the character of the milk agent, its

relationship to normal tissue constituents and its mode of action by combined biochemical, ultracentrifugation, and electron-microscopy technics is continuing (Graff *et al.*, 1952; Dmochowski and Passey, 1952). Results obtained from electron microscope examination of extracts and of ultracentrifugates of extracts of various tissues from high-mammary-cancer strains, supported by simultaneous biologic tests, indicate an association of cancer-inducing activity with typical particles mostly 200–300A in diameter (Dmochowski and Passey, 1952). Graff *et al.* (1952) have isolated much larger particles, with high density to the electron beam from milk of high-mammary-cancer mice and have concluded that these particles in high dilutions induce cancer, elicit antibodies in the rabbit, and are antigenically distinct from normal proteins of the mouse or mouse milk. In contrast, Dmochowski and Passey found serologic similarity between the typical particles in tissues of mice carrying the milk agent and extracts of tissues of agent-free mice. Tests designed to detect antigenic differences by the use of neutralizing antibodies, precipitins or complement fixation should include material from tissues, normal or cancerous, of both agent-free and agent-carrying mice which are genetically identical (Law and Malmgren, 1951).

It is quite obvious that more elaborate and detailed biologic testing of the isolated products is necessary in such studies to establish virus activity on a quantitative basis. Difficulties encountered in such work have been reported by Barnum *et al.* (1947, 1948).

C. *Genetic Control of the Propagation and Transmission of the Milk Agent.* The effect of the discovery of the mammary-tumor milk agent was to overemphasize the importance of this influence in the genesis of mammary tumors. Subsequent investigations have revealed the relationship of the milk agent to the genetic constitution; this relationship should be emphasized because of its importance to an understanding of the etiology of mammary cancer in mice and more generally to the probable intimate relationship of genetic factors and other pathogens. The observations of Murray and Little (1939), Fekete and Little (1942), and Andervont (1945c) suggested that the mammary tumor agent was not propagated in mice of their sublines of the C57BL strain nor in backcross mice in which C57BL chromatin was concentrated. Transmission of the agent to genetically susceptible young could not be detected (Andervont, 1945c). More direct evidence of the influence of genetic factors on the propagation and transmission of the milk agent was obtained by Heston *et al.* (1945). A detailed comparison of two groups of backcross females with comparable maternal influences, but unlike genetically, was made. These two groups of backcross females were developed by mating C3H strain females (more than 90% mammary tumors) to C57BL (B) strain males

(less than 1% mammary tumors), and in turn backcrossing the resulting F_1 hybrids (C3H x B) with (1) C3H males to obtain the susceptible strain backcross (F_1 x C3H), and (2) C57BL males to obtain the resistant strain backcross (F_1 x C57BL). Both backcross groups were obtained from genetically identical F_1 mothers having the mammary-tumor milk agent obtained from the C3H strain. Thus, it may be considered that both backcross groups had comparable maternal influences: the milk agent and possible cytoplasmic and intra-uterine factors, although the groups were unlike genetically. The susceptible backcross mice (F_1 x C3H) had, on the average, three-fourths chromatin from the C3H strain while the resistant backcross mice (F_1 x C57BL) had only one-fourth, on the average, C3H strain chromatin.

In order to determine the ability of these two groups to transmit the milk agent, individual females foster-nursed a genetically uniform group of test females, the F_1 progeny of C57BL strain females x C3H strain males. These test mice were susceptible to, but free of, the milk agent.

Comparison of the test females foster-nursed on these two groups showed that the two groups differed in their ability to transmit the milk agent, a significantly higher incidence of mammary tumors being found among the test mice nursed by the susceptible strain backcross females (Table IV). Since the test females were genetically uniform for both

TABLE IV

Evidence of Influence of Genetic Factors on Transmission of Mammary Tumor Milk Agent*

Foster Mother	Incidence of Mammary Cancer in Foster Mothers (%)	Number of Foster-Nursed Test Females (♀ C57BL x ♂ C3H)	Incidence of Mammary Cancer in Test Females (%)	Average Cancer Age in Months
Susceptible backcross (♀ F_1 x ♂ C3H)	93.2	375	89.6	14.9
Resistant backcross (♀ F_1 x ♂ C57BL)	55.1	355	67.9	15.0

* Data of Heston *et al.* (1945).

groups, this difference is attributable to the genotypic differences of the two backcross groups, strongly indicating genetic control over the propagation and transmission of the milk agent. The probability that the test females would develop mammary cancer thus was greatly influenced by the genetic constitution of the fathers of their foster mothers. Further experimentation is necessary to determine whether the differences are the

result of the amount of agent received, change in virulence, inactivation, neutralization, etc. From the results of work of a comparable nature in *Paramecium*, concerning the relationship of gene *K* to the killer substance kappa there has been found (Chao, 1952), a direct proportionality between gene dosage and the number of kappa particles. This would suggest a genic control of the maintenance, duplication, or level of the milk agent in the problem under discussion.

A detailed analysis made of the transmission of the milk agent by the individual females of both backcrosses revealed: (1) a highly variable transmission of the agent varying in degree from a highly effective transmission to no transmission, (2) transmission was more effective in backcross females which eventually developed mammary cancer, and (3) resistant females which did not develop cancer in many cases effectively transmitted the agent.

This latter observation suggests the operation of genetic factors which control the response of the mammary tissues to the milk agent as well as factors which govern its propagation and transmission. Recent observations of Heston (unpublished) support this hypothesis.

Although the evidence shows that genetic segregation in the backcross females influences in some manner the propagation and transmission of the milk agent, appropriate breeding tests of backcross segregants indicate that more than one gene pair is involved.

The intimate relationship of the genotype of the mouse to the mammary-tumor milk agent has its counterpart in two well-known phenomena of inheritance, the gene-inherited factor relationship in CO_2 sensitivity of *Drosophila* (L'Heritier, 1951) and the gene *K*-kappa relationship in *Paramecium* (Sonneborn, 1951). Illustration of how, in general, the mammary cancer problem fits the gene-cytoplasm relationship concept as developed in *Paramecium* has been given by Heston *et al.* (1945) and Sonneborn (1947). It seems pertinent to point out that there are basic differences between the phenomena observed in *Drosophila* and *Paramecium* and the mammary tumor problem: (1) The milk agent, as far as is known, is not in the germ line. The simple procedure of taking young by Caesarean section and foster-nursing frees a line of the agent. (2) Mutable forms of the milk agent have not been reported, while mutation in kappa and the inherited CO_2 sensitivity factor are known. Nevertheless, the general mechanism of nucleocytoplasmic relationship, as developed by Sonneborn, is instructive in attempting to answer some of the problems relating to the milk agent and mammary cancer: (1) the disappearance of the agent in some strains of mice, for example in BALB/c and $C3H_b$ following introduction of initially small doses; (2) the inability of some sublines of the C57BL strain to propagate and transmit the agent;

(3) the complete spontaneous disappearance of the milk agent in a strain of mouse, for example in the Marsh strain (Murray and Warner, 1947) and in the STOLI strain (MacDowell and Richter, 1935).

In considering the origin of the milk agent it would appear *a priori* that it is not similar to well-known plasmagenes, which appear to arise from a normal constituent of the cytoplasm. It apparently is not carried in the germ line. If it is a parasite of external origin, it is very perfectly adapted to the body cells, since it shows no pathogenicity, is very efficient in multiplication, relies on transmission, normally, through the mother's milk, and is strikingly influenced by the genotype. Andervont (1952) has investigated the origin of the milk agent in so far as its relationship to domesticated inbred mice is concerned. The following findings were reported: (1) wild mice were susceptible to the milk agent from an inbred strain, the C3H strain and successfully transmitted the agent through successive generations of offspring, (2) although the spontaneous incidence of mammary cancer in wild mice was low, appropriate test mice, the BALB/c and $C3H_b$ strains, showed an increase from 5 to 30% mammary cancer when fostered upon wild mothers. The descendants of fostered test mice were followed for five generations, and mammary cancer continued to appear in the line with evidence of virus enhancement in the later generations. These findings indicate that the appearance of the milk agent in certain inbred strains of mice is the result of selection during inbreeding of (1) mice originally carrying the agent and (2) genetically susceptible mice capable of propagating and transmitting it.

In considering the origin of the milk agent within the commonly used inbred strains, several interesting facts appear (Heston, 1949a). The high-mammary-cancer strains A, C3H, and DBA all have a common origin and all contain the agent. The CBA and BALB/c strains, which had their origin in this strain family, apparently do not harbor the agent but are genetically susceptible, so that introduction of the agent transforms these strains to high-mammary-cancer lines in which the agent propagates and is transmitted regularly to the offspring. The Marsh strain and the RIII strain, which also contain the agent, are not related, as far as known, to this strain family or to each other. Another distinct family of strains includes the C57BL, C57BR, C57L, and C58 strains, all of which appear to be free of the agent and express varying degrees of genetic resistance to the agent.

D. *Hormonal Mechanisms in Mammary Cancer.* The hormones associated with pregnancy and lactation will accelerate the development of mammary cancer. Table I shows typical differences between virgin and breeding females. The most pronounced effect is found in the A strain in which females maintained as virgins rarely get mammary cancer

whereas females subjected to prolonged hormonal stimulation of mammary tissue reveal a high incidence. Jones (1940) has shown that the incidence in the A strain increases with the number of pregnancies. Similar differences in other strains are less striking. In the C3H strain the incidence in virgin and multiparous females is the same, but mammary cancer appears much later among the virgins. Genetic differences among the different DBA strain sublines probably account for the variation observed in different laboratories. That hormonal stimulation and not pregnancy per se is responsible for the differences observed in the A strain has been shown by Law (1941), who noted an increase in the incidence of mammary cancer in virgin females following pseudo-pregnancy.

Direct evidence for the hormonal influence has been obtained in several ways: (1) Early ovariectomy inhibits or strikingly delays mammary cancer formation, depending on the strains used. An exception has been observed by Woolley *et al.* (1940, 1943) in the DBA strain. Postcastrational hyperplasia of the adrenal cortex occurs in DBA strain females ovariectomized at one day of age; mammary cancer in these mice probably results from the hormonal stimulation thus provided. (2) Mammary cancer appears in male mice, particularly castrates, and in virgins following administration of synthetic estrogens, provided the milk agent is present. Approximately the same incidence is observed in castrated males and virgins, given estrogens, as is encountered in multiparous females of the strain.

It has been suggested that differences in the production of estrogenic hormone might form the basis for the differences in incidence of mammary cancer among the various inbred strains. A difference in threshold sensitivity of mammary tissue to neoplastic change might also explain the strain differences. Although striking differences in the characteristics of of the estrus cycle have been observed (Loeb and Genther, 1928; Lacassagne, 1934; Burns *et al.*, 1936) and are believed to be genetically determined (Armstrong, 1948), the differences do not seem to bear a specific relationship to the incidence of mammary cancer.

Differences in vaginal response to estrogens have been reported by Van Gulik and Korteweg (1940) and Shimkin and Andervont (1941). In each experiment the high-mammary-cancer strains were much less sensitive than low-cancer strains. That these differences are in fact strain differences only and not causally related to mammary cancer was shown by Shimkin and Andervont, who changed the cancer incidence of the high-cancer strains by foster-nursing without influencing vaginal sensitivity to estrone. While the vaginal response of the DBA and C57BL strains of mice differed considerably, sensitivity of the mammary tissue of ovariectomized females showed no differences (Mühlbock, 1948).

The hormonal mechanism, whatever its nature, responsible for the high incidence of mammary cancer in virgin mice of the C3H strain has been shown by Bittner *et al.* (1944) and Heston and Andervont (1944) to be under genic control. Reciprocal crosses were made between the C3H strain, having an incidence of mammary cancer greater than 90% in both virgin and multiparous females, and the A strain a high-cancer strain in which virgin mice have strikingly few mammary cancers (see Table V).

TABLE V
Influence of Genetic Factors on Hormonal Mechanism*

Virgin Females	No.	Per Cent Mammary Cancer	Mean Tumor Age (months)
A	22	0	—
C3H	29	93	10.9
AC3H F_1	45	91	15.8
C3HA F_1	36	83	13.8

* Data of Heston and Andervont (1944).

The incidence of mammary cancer in the reciprocal F_1 hybrids was the same, within limits of error. Of particular interest is the cross of A strain females and C3H strain males, which shows that the introduction of C3H strain chromatin increased the mammary cancer incidence from 0%, characteristic of the A strain virgins, to 91%, characteristic of the C3H strain virgins. Reciprocal foster-nursing experiments, the A strain receiving C3H milk agent and the C3H strain receiving A milk agent ruled out any effect of this agent. Thus, it is apparent that a change in the genetic constitution has resulted in an increase in the incidence of mammary cancer in F_1 virgin mice. It is probable that genetic factors in some manner control some phase of hormone production or metabolism or response of mammary tissues to hormone stimulation.

The conclusion that genetic factors are responsible for the high incidence of mammary cancer in F_1 hybrids of the cross ♀ A x ♂ C3H was further strengthened by the observation of Bittner and Huseby (1946) that segregation of genes influencing susceptibility occurred in the F_2 hybrid generation. The observations of Bittner (1944; Bittner and Huseby, 1946), contrary to the findings of Heston and Andervont (1944), indicate a difference in the concentration and/or activity of the milk agent in A strain and C3H mothers. The difference in incidence of mammary tumors observed by these investigators in the A and C3H strains indicate distinct subline differences in their material.

The presence of genetic factors, described above, which produce a high incidence of mammary cancer in virgin F_1 hybrid mice having an A strain

mother have been described as being present in addition to the C3H strain in the DBA strain, sublines 8 and 2, the BALB/c strain and the I strain. (Bittner, 1952). Whether these strains have genetic factors in common remains to be determined. From the data at hand, it would appear that more than one pair of genes is responsible for the differences described.

While it is clear that the A and C3H strains differ fundamentally in the genic complex influencing mammary cancer development, the physiological mechanisms involved require further study. Though it would appear *a priori* that the differences are simply those of ovarian secretion, since virgin A strain females given estrogens develop mammary cancer (Gardner, 1939; Suntzeff *et al.*, 1936) and A strain breeding females show a high incidence, a simple, clear-cut explanation has not been established. Deringer *et al.* (1945) have observed that the estrous cycles of these 2 strains of mice and their F_1 hybrids were strikingly similar, but that the vaginas of A strain females opened 10 days later. Huseby *et al.* (1946) and Huseby and Bittner (1950), in an attempt to characterize the physiological mechanisms involved, transplanted ovaries from the A and C3H strains and from AC3H F_1 hybrids into overiectomized F_1 hybrids between the 2 strains. The variables involved were reduced to those resident in the ovaries of the donor mice, since these would be subjected to the same pituitary control and the transplanted ovaries would be acting upon genetically identical end-organ substrates. Though no differences among the groups were found by the vaginal smear technic, the F_1 mice bearing C3H and F_1 ovaries had a somewhat higher incidence of mammary cancer, appearing earlier, than those bearing A strain ovaries. The authors attribute this to a difference in the "carcinogenicity" of A and C3H ovarian secretions. That the differences in the physiologic nature of the hormonal patterns of A and C3H mice may be subtle and difficult to identify by ordinary technics is indicated by the work of Smith (1945). Ovariectomized C3H strain and C3HA F_1 hybrid mice developed adrenal cortical hyperplasia with a consequent cornification of vaginal epithelium, uterine hypertrophy, and mammary gland development while no histologic changes were noted in adrenals of A strain mice. It is apparent that much work remains to be done on such fundamental and important findings relating to the etiology of mammary cancer.

E. *Genetic Susceptibility in Relation to Mammary Cancer.* It has been shown that genetic factors govern the hormonal mechanism and the propagation and transmission of the mammary-tumor milk agent. The genotype of the mother and foster mother are known also to exert a control over the transmission of the milk agent. It is clear also that genic differences may be expressed through sensitivity of the mammary tissue

to various stimuli particularly the ovarian hormones and the milk agent although this latter group of genetic factors have not been adequately characterized to date. Genetic analysis of mammary cancer is seriously complicated by strong intrinsic influences such as the milk agent and estrogenic stimulation both of which are under genetic control and by various other factors such as age of mother, the litter in which mice were born, early or late, etc. In view of the difficulties inherent in a genetic analysis of the influences operative in mammary cancer, it is clear that decisive evidence will be obtained only under the most stringently controlled conditions.

The early work of Bittner (1940) indicated that susceptibility to mammary cancer was inherited as a single dominant factor. It is clear (Bittner, 1944; Heston, 1944) that such a simple interpretation is inadequate. The presence of the milk agent in certain experimental situations, where it was thought ineffective, is now known to preclude a direct measure of the effect of genotype alone.

Andervont (1945a), Foulds (1949), and Bittner (1950) have observed the appearance of a high incidence of mammary cancer in some F_1 hybrids obtained by crossing milk agent-free females with males from a high-cancer line. Heretofore, the influence of the high-cancerous father has generally been regarded as proof for the existence of genes which influence susceptibility to cancer, since it was difficult to imagine the male contributing any influences other than genes to the offspring. F_1 females, in experiments involving crosses between BALB/c females and C3H males, have shown an incidence of mammary cancer of 60%, greatly in excess of the incidence in BALB/c mice and more nearly that of the high-cancer strain. Both Andervont and Foulds have shown that some of the F_1 females possessed the milk agent and transmitted it to their offspring. The most probable explanation for this phenomenon is that the F_1 females acquired the agent through infection by the sperm (Andervont and Dunn, 1948). Thus, the validity of interpretations must be questioned ascribing increases in the incidence of mammary cancer in F_1 hybrid mice to chromosomal factors introduced by the male. The general nature of extrachromosomal influences supplied by the male remains to be determined.

The influence of genotype, in the absence of the mammary-tumor milk agent, has been studied recently by Heston *et al.* (1950). A line of mice $C3H_b$, free of the milk agent, was developed by removing C3H strain mice by Caesarean section and foster-nursing upon an agent-free strain, C57BL. These mice were then maintained by sib matings. The following observations and conclusions are significant: (1) A relatively high incidence of mammary cancer may be obtained in the absence of the milk agent. Breeding females had an incidence of mammary cancer of 38% at

an average age of 20 months. (2) Contrary to most of the observations in the literature indicating complete lack, or nearly so, of mammary cancer, under the proper experimental conditions mammary cancer will appear in the absence of the milk agent. (3) A strong genetic susceptibility along with estrogenic stimulation, as a result of intensive breeding, had a combined effect sufficient to exceed a physiologic threshold leading to neoplastic transformation.

These observations indicate that none of the well-recognized influences in the induction of mammary cancer in mice: (1) genetic susceptibility, (2) estrogenic stimulation, or (3) the virus-like milk agent are to be considered as primary causative influences. The action and interaction of all, and in certain circumstances a combination of two, increase the probability that mammary cancer will occur. To date, no one influence has been shown to result in an all or none difference under any set of conditions.

Table VI shows, in a comparative manner, the results of varying any one of the three major influences while holding the others constant. The effect of the milk agent, measured when other influences are held constant, may be seen as the difference between breeding females of the C3H strain and the $C3H_b$ line, a reduction of 59% in the incidence of mammary cancer. This difference is more striking in comparing the incidence in virgin females: 97% for C3H and 4% for $C3H_b$. The difference in incidence due to the milk agent upon the C57BL genetic background is only 14%. Introduction of the milk agent from the C3H strain into BALB/c mice converts this strain to a high-mammary-cancer strain, maintaining this high incidence in the progeny indefinitely.

The most pronounced effect of intrinsic estrogenic stimulation is seen in A strain mice, a difference in incidence between virgins and multiparous females of nearly 80%. In the C57BL strain, too few mammary cancers appear to detect a difference and in the C3H strain the incidence in virgins and breeding females is identical except that the latent period is longer by 2 months in the virgins. The response to estrogenic stimulation in $C3H_b$ mice, in the absence of the milk agent is measurable, virgin females having 33% less mammary cancer than breeding females.

The effect of genetic factors may be seen by comparing the C3H and C57BL strains. In breeding females, both groups having the C3H milk agent, the difference in the incidence of mammary cancer is 81%. In the absence of the milk agent, the effect is represented in the difference between the $C3H_b$ and C57BL breeders, 38%. The difference in incidence between the A and C3H strains, both high-cancer strains, is probably genetic. This difference persists in the case of reciprocal foster-nursing (Heston and Andervont, 1944).

The genetic differences observed in crosses between high- and low-mammary-cancer strains are not simply due to a single gene. The variation in hybrid generations is continuous indicating a similar and supplementary effect of several genes. That physiological thresholds may bisect

TABLE VI

Incidence of Mammary Cancer in Several Homogeneous Strains of Mice as Result of Varying Factors: Milk Agent, Estrogenic Stimulation or Genetic Susceptibility

Milk Agent		Estrogenic Stimulation	Genetic Susceptibility	Incidence of Mammary Cancer	Reference
a. Varying milk agent					
C3H	+	+	+	97	Heston *et al.* (1950)
$C3H_b$	− (fostered C57BL)	+	+	38	
C57BL	−	+	−	0.0	Andervont (1940a)
$C57BL_Z$	+ (fostered C3H)	+	−	14	
BALB/c	−	+	+	1.4	Andervont (1945a)
BALB/c	+ (fostered C3H)	+	+	70	
b. Varying estrogenic stimulation					
C3H	+	+	+	91.4	Andervont (1941)
C3H	+	−	+	97.4	
$C3H_b$	−	+	+	38	Heston *et al.* (1950)
$C3H_b$	−	−	+	4	
A	+	+	+	83.6	Bittner (1939a)
A	+	−	+	4.9	
C57BL	−	+	−	0.5	Little & Pearsons
C57BL	−	−	−	0.0	(1940)
c. Varying genetic susceptibility					
C3H	+	+	+	92	Bittner (1940)
$C57BL_Z$	+ (fostered C3H)	+	−	11	
$C3H_b$	− (fostered C57BL)	+	+	38	Heston *et al.* (1950)
C57BL	−	+	−	0.0	
C3H	+	−	+*	94	Heston & Andervont
A	+ (fostered C3H)	−	−	16	(1944)

* A difference in genetic susceptibility between the A strain and C3H strain mice maintained as virgins is discussed in the text. Reciprocal foster nursing between these strains did not give conclusive evidence of a difference in the milk agent of the two strains.

continuous variation so as to simulate Mendelian segregation in certain situations was shown in the observations of Bittner (1940) and Andervont (1937a). The multifactor interpretation gains further support from the incidence of linkage between susceptibility and the yellow gene (A^y) as shown by Little (1934), the gene determining brown coat color (*b*)

(Bittner, 1945), and the agouti gene (A) (Heston and Deringer, 1948). (See Fig. 1.)

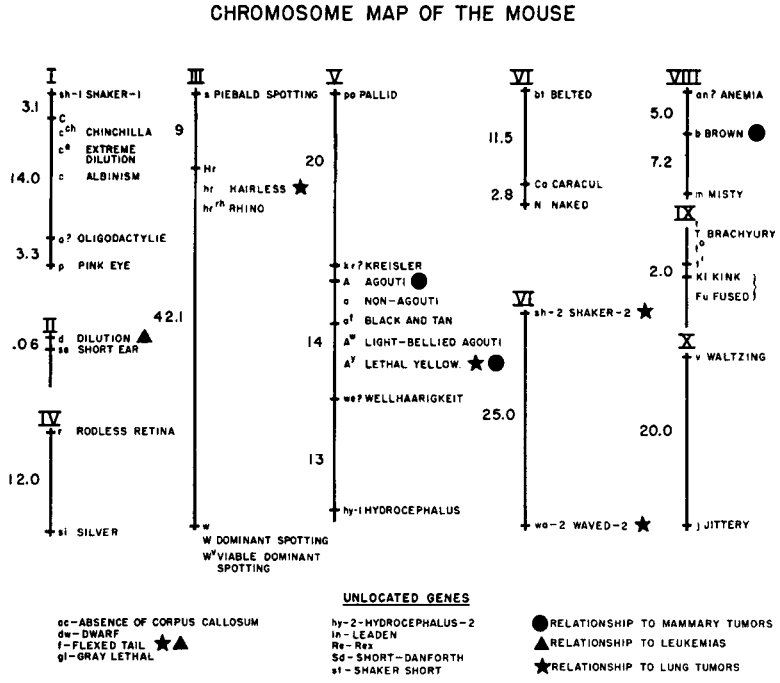


FIG. 1. Association of specific marker genes in the mouse with susceptibility to mammary cancer, pulmonary tumor, and leukemia.

2. Pulmonary Tumors

A. *Genetic Studies.* The primary tumors of the lung are considered to be epithelial in origin. In the mouse it is believed that the pulmonary growths arise from alveolar lining cells (Grady and Stewart, 1940), while the common pulmonary tumors in man, in contrast, appear to have their origin from bronchial epithelium. Under certain experimental conditions, however, it is possible to reproduce in mice most of the histologic types found in man (Andervont, 1937b; Smith, 1952). Cloudman (1941b) distinguishes several histologic types of pulmonary tumor in the mouse: adenoma, adenocarcinoma, papillary adenocarcinoma, carcinoma simplex, and carcinosarcoma. The common pulmonary tumor of the mouse, alveologenic carcinoma, occurs in either lung and any lobe situated close to the pleura so that it may be seen easily on the surface. Induced tumors are, for the most part, multiple and are indistinguishable histologically from spontaneous tumors. The common lung tumors are not encapsulated,

metastasize on occasion (Wells *et al.*, 1941) and will grow progressively upon transplantation into appropriate mice.

Table VII presents the incidence of pulmonary tumors in some inbred

TABLE VII
Incidence of Pulmonary Tumors in Some Inbred Strains of Mice

Strain	Incidence of Pulmonary Tumors (%)	Other Characteristics
A	80–90*	Approximately 80 % mammary cancer
C3H	5–10	90 % mammary cancer; 10–30 % hepatomas
C57BL	<1	Less than 5 % mammary cancer
C57L	<1*	
BALB/c	20–30	
Swiss	40–50	
C58	<1	90 % lymphocytic leukemia
I	10–20	Adenomatous lesions of stomach

* Carcinogenic hydrocarbons induce 100 % pulmonary adenomas in A strain mice at 6 weeks of age with 75 lung nodules per mouse; whereas in the C57L strain only 25 % pulmonary adenomas are found at 48 weeks with 0.27 lung nodule per mouse.

strains of mice commonly used. Spontaneous pulmonary tumors appear later in life than mammary cancer, and time of onset is difficult to determine. Histologically identical tumors may be induced by the administration of various chemical and physical agents, such as carcinogenic hydrocarbons (Murphy and Sturm, 1925; Andervont, 1937c), urethane (Nettleship and Henshaw, 1943), nitrogen and sulfur mustards (Heston, 1949b, 1950), and gamma rays (Lorenz *et al.*, 1946). Those strains which show a high spontaneous incidence of pulmonary tumors (A, C3H, Swiss) are the most sensitive to induced tumors as indicated by early appearance, number of mice affected, and number of individual lung tumors. This response of susceptible mice has been made use of in simplifying genetic studies, as will be discussed, and as the basis for a most sensitive test of carcinogenicity (Andervont and Shimkin, 1940; Heston, 1949b). Since susceptibility to induced pulmonary tumors parallels susceptibility to spontaneous tumors, it appears that the carcinogens merely act by accelerating a process of transformation which is to occur under normal circumstances. This effect resembles that of estrogens in the development of mammary cancer, but is unlike the relationship of carcinogens and gamma rays in the induction of leukemia, which will be discussed later.

The genetics of pulmonary tumors was first investigated by Lynch (1926), using strains of mice that were not completely homogeneous. Her work indicated that susceptibility to spontaneous pulmonary tumors was largely under genetic control and that susceptibility was probably domi-

nant. In conformity with the findings of Lynch, Bittner and Little (1937) and Bittner (1938) concluded from studies of crosses between the high-tumor A strain and the C57BL strain, in which pulmonary tumors are rare, that susceptibility was probably transmitted as a single dominant Mendelian factor. In contrast to the situation in man, no sex differences were found in reciprocal crosses nor was there any evidence of a maternal influence.

Andervont (1937a, 1938a,b), by the use of the carcinogenic hydrocarbon 1,2,5,6-dibenzathracene, increased the incidence of pulmonary tumors and strikingly hastened their appearance in crosses involving the A and C57BL strains. Ninety per cent of the F_1 mice from reciprocal matings developed lung tumors, 74.7% of the F_2, 100% of the backcross generation produced by mating the F_1 to the susceptible A parent, and 45% of the backcross generation produced by mating the F_1 to the resistant C57BL parent. These results were similar to those reported by Bittner and suggested that a single dominant factor may be involved in the inheritance of susceptibility to pulmonary tumors.

In analyzing susceptibility to tumors, it is questionable that classifying the animals in alternate categories, lung tumor or no lung tumor, will reveal the true genetic situation. Possible variation in the F_2 and backcross generations is concealed and there is no means for determining the influence of nongenetic as well as genetic factors. The critical experiment is to test the genetic nature of the types segregating in the backcross or the F_2 generation by breeding with the resistant strain. This has not been done. It has been shown by Wright (1934), for example, in polydactyly in the guinea pig that although the ratios obtained simulated ratios suggesting a single major gene difference, actually at least 4 genes were involved.

Even though segregation by ratios of types may not be followed in Mendelian fashion, it is still possible to detect its occurrence by the biometrical properties of frequency distributions as first proposed independently by Nilsson-Ehle (1909) and East (1915). Investigations of this sort have been reported by Heston (1940–1942), who used two criteria as a measure of the degree of susceptibility, (1) the time required for tumors to develop following injection of a potent carcinogen (latent period) and (2) the number of tumor nodules per individual. With the aid of these quantitative measures of susceptibility, crosses were made between the highly susceptible A strain and three resistant strains, C57L, N and W. The F_1 hybrid animals were found to be intermediate between the parental strains, and the backcross to the resistant strain in each case was intermediate between the F_1 and the resistant strain. Variance in the F_2 generation and in the backcross generations was considerably

greater than in the parental strains and the F_1 hybrid generation (see Fig. 2). This is to be expected in generations in which segregation occurs. Since all these individuals will not be alike genetically (unlike the parental strains and F_1), genetic variation is added to nongenetic variation, increasing the variance of these groups. In none of the segregating generations was there any sign of bimodality in the variation. A more complete analysis was made of crosses between the A (high) and C57L (low) strains

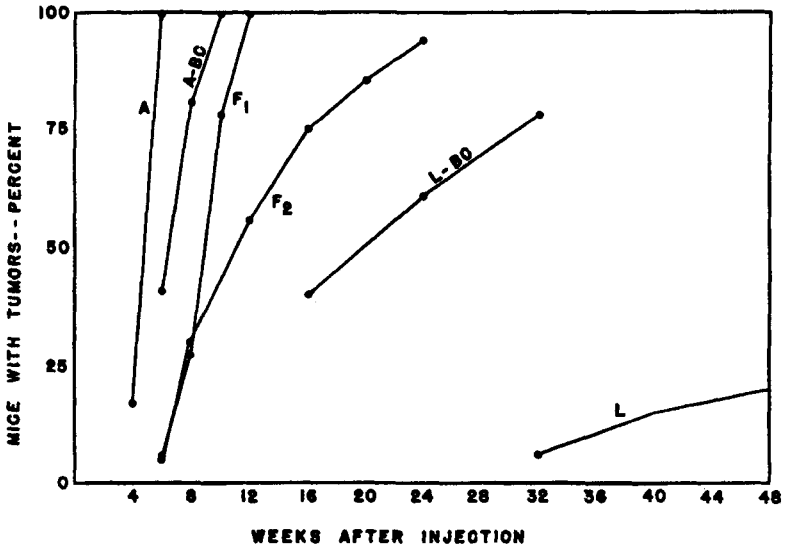


FIG. 2A. Pulmonary tumor incidence in the A and C57L strains and in the F_1, F_2, and backcross hybrids. Mice were sacrificed at various intervals following intravenous injection of 1,2,5,6-dibenzanthracene (data of Heston, 1942).

(Heston, 1942a) (see Table VIII). By measuring the variance in the various hybrid generations, in which segregation of genes would be expected, it was found that approximately 14% of the total variance was the result of nongenetic influences and 86% could be attributed to genetic factors. A rough estimate of the least possible number of genes involved was four. It would appear, then, that the inheritance of susceptibility to induced pulmonary tumors is determined by several genetic factors with additive effects similar to the inheritance of such other quantitative characters as size and corolla length.

B. *Effect of Specific Genes and Site of Action.* With the demonstration that the specific genes which are known to influence induced pulmonary tumors can also be shown to have a comparable influence upon the development of the spontaneous type (Heston and Deringer, 1947, 1949), it is apparent that the mechanism analyzed by the use of induced tumors

applies equally as well to spontaneous tumors, at least so far as tumors of the lung are concerned. The carcinogens may therefore be considered potent nongenetic factors whose effect will depend on the genetic susceptibility of the strain.

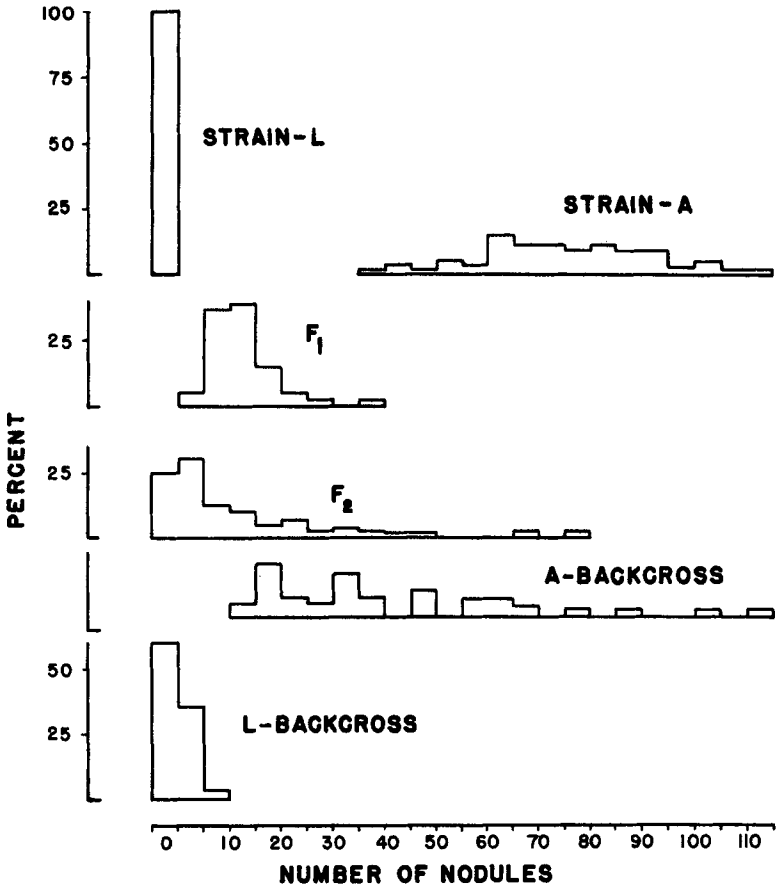


FIG. 2B. Frequency polygons showing number of tumor nodules in groups of the A and C57L strains and hybrid mice at 16 weeks after injection of carcinogen (data of Heston 1942).

Many experiments which show multifactor inheritance also reveal linkage of these genes and major genes, that is, those segregating in Mendelian fashion. In the case of some such genes it may be difficult to detect linkage because of their small effect. Pulmonary tumors seem particularly adapted to linkage studies probably because of the delicate quantitative measure employed. Susceptibility to induced lung tumors is

influenced by genes located in linkage group VII (genes *wa-2* and *sh-2*) and in the chromosome carrying the gene, flexed-tail (*f*) (Heston, 1941). It has not been determined definitely whether susceptibility is influenced by these genes themselves, or by genes linked to them although observations of Heston *et al.* (1952) indicate an influence of the flexed-tail gene per se. In each case there was found to be a decrease in susceptibility. The gene for Yellow (A^y), linkage group V, increased susceptibility to lung tumors, although in the cross used this gene was introduced from the

TABLE VIII

Induction of Pulmonary Tumors in A and C57L Strains of Mice and in F_1, F_2 and Backcross Hybrids, 16 Weeks after Intravenous Injection of 1,2,5,6-Dibenzanthracene*

Strain or Hybrid	No. of Mice	Per Cent Mice with Tumors	Mean No. of Nodules per Individual	Standard Deviation
A	108	100	75.4	15.7
C57L	22	0	0	—
F_1	103	100	12.5	5.3
F_2	105	75	10.0	14.1
A-Backcross	41	100	41.9	24.3
C57L-Backcross	30	40	0.7	1.3

* Adapted from data of Heston (1942a).

resistant strain (Heston, 1942c; Heston and Deringer, 1947). More recently, another gene hairless (*hr*), linkage group III, has been shown to reduce susceptibility (Heston and Deringer, 1949). (See Fig. 1.) Thus, it is clear that susceptibility to lung tumors is influenced by genes located on at least four chromosomes; this is in agreement with the multifactor interpretation of inheritance.

Recently Heston *et al.* (1952) have shown a relationship between the several genes which decrease susceptibility to pulmonary tumors and body weight. The genes flexed and shaker-2 significantly reduced body weight while the gene for pink eye (*p*) did not. These results are in accord with an earlier report of Morgan (1950), showing that heavier yellow mice of an inbred strain Y, were more susceptible to pulmonary tumors than their non-yellow sibs. These results indicate a relationship between normal growth processes and pulmonary tumors.

In an effort to determine whether the action of genes controlling the development of pulmonary tumors is localized in the lung tissue or is manifested through some general systemic mechanism, lung tissue from A strain mice, highly susceptible to pulmonary tumors, and from C57L

mice, highly resistant to pulmonary tumors, were transplanted subcutaneously into a common host, the F_1 hybrid of the two strains in which most normal tissues will maintain themselves indefinitely. Pulmonary tumors were induced in 39% of the lung fragments derived from the A strain and in 3.6% of the C57L fragments, following intravenous injection of the carcinogen 1,2,5,6-dibenzanthracene. Thus, the fact that the difference in degree of genetic susceptibility in A and C57L lung tissue persisted when grown in a common host indicates strongly that the action of most of the genes by which these two strains differ is confined to lung tissue (Heston and Dunn, 1951). Similar results in other strains have been presented by Shapiro and Kirschbaum (1951).

3. Leukemia

Most of the neoplasms of non-epithelial origin encountered among the spontaneous tumors of the mouse originate from blood-forming tissues. The various forms of leukemia which are found in man are imitated to a surprising degree in the mouse. It is becoming obvious that the leukemias of man and lower animals, particularly the mouse, are analogous (Furth *et al.*, 1935; Engelbreth-Holm, 1942). The unquestionable neoplastic forms in man: lymphocytic, granulocytic, monocytic (reticulum cell sarcoma, reticuloendothelioma, histiocytoma, etc.) mast cell, and plasma cell leukemia have been described in the mouse. In addition, lesions appearing in old mice, especially in strain C57L and some leukemic strains, or in hybrids having one parent of a leukemic strain, have been described which involve the reticulum cells and which appear also to be of neoplastic nature. These lesions have been termed Hodgkins-like lesions. Histologically they have a considerable resemblance to Hodgkins disease of man (Dunn, 1951, 1953). For details concerning the comparative pathologic aspects of this complex group of neoplasms the reader is referred to Engelbreth-Holm (1942) and Dunn (1951, 1953).

The first description of leukemia in mice was given by Eberth (1878) and was followed by many isolated reports describing the typical leukemia of the mouse, the lymphocytic form. It was not until the development of homogeneous strains of mice that any real advances were made in our knowledge of the disease in the experimental animal. Although many early attempts were made to transmit leukemia in mice of heterogeneous stocks (Tyzzer, 1907; Haaland, 1911; Levaditi, 1914) it was not until 1929 that Korteweg succeeded in transplanting, in consecutive transfers, an induced lymphosarcoma which appeared in a mouse of a homogenous strain.

A. *Genetic Studies.* The development of the high-leukemic C58 strain of mice by MacDowell (Richter and MacDowell, 1929; MacDowell and Richter, 1930) and subsequent studies using this strain (MacDowell,

1929–1952) have established the genetic basis of susceptibility to spontaneous leukemia. It was observed (MacDowell and Richter, 1935) that though inbreeding, brother x sister, resulted in a common genetic constitution of the C58 strain which predisposed to leukemia, only 90% of the animals ever developed the disease. It was inferred that the differences represented by the 90% leukemics and 10% nonleukemics were not genetic differences. That nongenetic influences in fact accounted for the 10% nonleukemics in the strain was shown by (1) a random distribution of nonleukemics among the different families and branches of the pedigree and (2) offspring obtained by mating nonleukemic mice gave the same incidence of leukemia as offspring obtained from leukemic parents:

C58 Parents	C58 Offspring		Incidence of Leukemia
	+	−	
+ x +	286	36	88.8%
+ x −	55	5	91.7%
− x −	10	−	100.0%

In crosses of the genetically homogeneous C58 strain with a low-leukemic strain, Storrs-Little (STOLI), in which leukemia is rare (see Table IX), it was found by MacDowell and Richter (1935) that (1) the

TABLE IX

Incidence of Leukemia in Various High- and Low-Leukemic Strains of Mice

Strain	Per Cent Leukemia	Mean Length of Life of Leukemics (days)	Remarks
C58	90.5	360.3	Incidence similar in both sexes
BALB	68.0	679.8	Typical forms not specified
AK	58–80	293	Slightly higher incidence and earlier occurrence among females
AKR*	85	232	Incidence similar in both sexes. 97% lymphocytic leukemia
F	53	420	13% granulocytic leukemia. All others either lymphocytic or mediastinal lymphosarcoma. No sex differences.
DBA/2	36	—	Lymphocytic leukemia
STOLI	1.3	688	
C_3H or C_3Hb	<1	516	
NH	<1	—	
Rf	1.6	—	

* Formerly called the RIL strain.

incidence of leukemia was intermediate between that of the parent strains, lymphocytic leukemia predominating, and (2) a specific maternal effect was observed. F_1 offspring obtained from C58 (high-leukemic)

mothers had an incidence of leukemia 19.4% higher than offspring from STOLI (low-leukemic) mothers, and (3) a nonspecific maternal effect lengthening the lives of both leukemic and nonleukemic F_1 mice was apparent in the cross using STOLI mothers. The difference in incidence observed in these reciprocal crosses was found equally in both sexes and thus cannot be due to sex linkage. Since the mice in the F_1 generation are genetically alike, the presence or absence of leukemia in a particular mouse must be the result of nongenetic influences. Again, the genetic uniformity of the mice is shown by comparing the offspring of leukemic and nonleukemic F_1 hybrid mice backcrossed to the low-leukemic STOLI strain:

F_1 Hybrid Parents	Offspring		Incidence of Leukemia
	+	−	
+	37	37	50%
−	16	21	43.2%

The specific maternal effect was found to be more pronounced in backcrosses of F_1 hybrids to the low-leukemic STOLI strain, the crosses of ♀ STOLI x ♂ F_1 showing a 27% lower incidence of leukemia than the reciprocal mating (see Table X). The mechanism of this maternal trans-

TABLE X

Incidence of Leukemia in Reciprocal Matings of High- and Low-Leukemic Strains of Mice

	Mating Combination H/L*					
	C58/STOLI	AK/Rf	AK/C3H	DBA/WA	AKR/NH	C58/C3Hb
	Authors					
	1 MacDowell & Richter (1935)	2 Cole & Furth (1941)	3 Furth *et al.* (1942)	4 Law (1952a)	5 Law (1953)	6 Law (1953)
	Incidence of leukemia (%)					
High-leukemic strain	89.6	69.3	58	69.7§	85.0	90.0
♀ H x ♂ L	61.9†	21.9‡	50‡	38.5	53.3	45.2
♀ L x ♂ H	42.5	11.6	34	19.7	33.5	41.1

* H, high-leukemic strain; L, low-leukemic strain.

† The maternal influence was even more pronounced in backcross mice, the cross ♀ STOLI x ♂ F_1 giving 19.8% leukemia and the reciprocal cross, ♀ F_1 x ♂ STOLI, 46.5% leukemia.

‡ These differences in reciprocal crosses were found in males only. See text.

§ Leukemia induced in these studies with methylcholanthrene.

mission was not demonstrated but it was suspected either that the cytoplasm of the egg was involved or that some influence was transmitted through the placenta during gestation. The influence of the mother's milk, similar to the effect observed in mammary cancer, was said to be eliminated, since reciprocal foster-nursing experiments failed to increase or decrease leukemia in the respective C58 and STOLI strains. No data were given however.

In an attempt to evaluate the precise role genetic factors play in determining susceptibility to leukemia, a series of hybrid crosses were made by MacDowell and Richter (1935) using the C58 and STOLI strains of mice and by Cole and Furth (1941) using the AK (high-leukemic) and Rf (low-leukemic) strains. The significant findings in both reports were that something influencing the incidence of leukemia was received from the parents, and the strength of this influence varied with the grandparents. In these experiments the proportion of leukemics in any given generation was a mathematical function of the total heredity contributed by the high-leukemic strain. In the experiments with the C58 and STOLI strains this function appeared to be arithmetical, whereas in crosses of the AK and Rf strains it was semilogarithmic. These differences probably result from differences in nongenetic variables imposed upon strain (genetic) differences.

Mercier (1937), in a study of crosses between a strain of mice having a relatively high incidence of lymphosarcoma (49.2%) and a low-leukemic strain and Slye (1931) in pedigree studies of mice of unknown ancestry, concluded that susceptibility to leukemia was inherited as a Mendelian recessive. It is clear, however, that these studies as well as those of MacDowell and Richter and Cole and Furth do not distinguish between chromosomal or extrachromosomal contributions by the parents. As shown above in regard to leukemia, it is apparent that the genetic constitution is not always expressed by whether or not a mouse gets leukemia; leukemic and nonleukemic C58 mice are genetically alike, yet two leukemic mice may be quite different genetically. The problem, in a genetic analysis, is to determine whether the mice in a generation in which reassortment of genes is occurring are uniform or diverse in their potentialities to produce leukemia (or cancer). If one assumes, for example, the presence within an inbred high-leukemic strain of a nongenic, cellular pathogen, this would in all likelihood be maintained at a uniform level in a strain by inbreeding but be reduced to lower levels by crosses with other strains. Thus it would be expected that all the individuals in a second hybrid generation, for example a backcross, would have uniform potentialities of developing leukemia. However, if genes were responsible for differences between high- and low-leukemic strains, the mice in a second

hybrid generation would be a genetically diverse group since there occurs reassortment of genes in crossbreeding, depending upon the distribution of chromosomes.

With this plan in mind, of determining whether the mice in a second generation of a cross between two strains differing in their potentialities to develop leukemia, are genetically uniform or diverse, MacDowell *et al.* (1945) set up an experiment to classify each animal in a generation in which segregation occurs. The classification was based upon the incidence of leukemia among the offspring of animals obtained in a backcross to the low-leukemic strain and not on a plus-or-minus description of the individual animal. Under these conditions, significant differences in the incidence of leukemia among families would constitute evidence of the segregation of genes influencing the appearance of leukemia. A bimodal distribution of families would indicate the predominant effect of a single gene (see Law *et al.*, 1952). If more than one gene were involved, a continuous variation may be expected in the distribution of families.

Analysis of the backcross mice was simplified by using a single C58 high-leukemic male. F_1 hybrids were produced by crossing with three females of the low-leukemic STOLI strain. The maternal influence, noted previously, was eliminated by transmitting susceptibility to leukemia only through the male line. The genotypes of fifty backcross males, obtained by crossing F_1 hybrids with the low-leukemic STOLI strain, were then characterized. A family of 50 second backcross mice was obtained for each male by crossing again with the STOLI strain, and the incidence of leukemia within each family was obtained. It may be seen in Fig. 3 that the observed proportions of leukemics in these 50 different backcross families varied from 0 to 42.8% in a fairly symmetrical frequency distribution with the modal class at 17%. This distribution indicates the existence of many different genotypes among the 50 males and shows that segregation is occurring for genes influencing susceptibility to leukemia.

An analysis of the numerous environmental variables influencing their experimental findings was given by MacDowell *et al.* in this study. For example, it was found that longevity was influenced by the genetic constitution of the father, by parturition age of the mother, by the strain of the nursing female, and by sex, each apparently acting in a different manner. This work emphasizes the extreme complexity of any genetic analysis, even employing the most highly homogeneous material available, when influenced to any degree by nongenetic variables.

B. *The Maternal Resistance Factor (MRF).* The existence of a maternal influence led to a search for a milk factor similar to that of the mammary-tumor milk agent, a tumor-inciting agent. Cole and Furth (1941) and

Furth *et al.* (1942), in crosses between the leukemic AK strain and low-leukemic Rf and C3H strains, reported reciprocal differences in their F_1 hybrid mice. It is to be noted that significant differences were found among the male mice only in both crosses, 28.1% leukemia compared with 8.8% in the cross using Rf low-leukemic strain, and 54% compared with 28% using the C3H strain. In the latter cross, interpretations were difficult because of the introduction by C3H females of the mammary-tumor milk agent. Reciprocal foster-nursing experiments between the AK

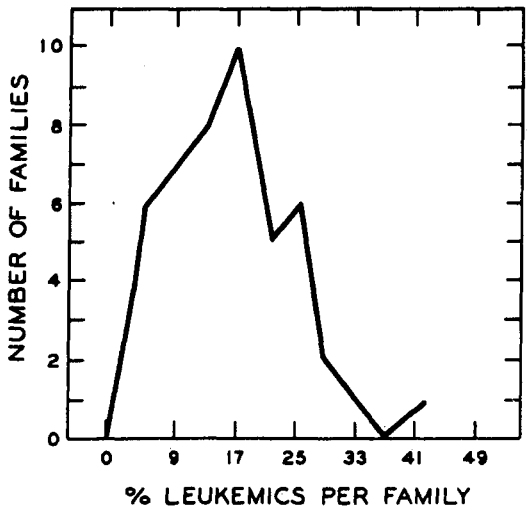


FIG. 3. Frequency distribution of second backcross families according to total incidence of leukemia (data of MacDowell *et al.*, 1945).

and C3H strains showed a reduction in incidence of leukemia in the high-leukemic strain but no influence on the incidence of leukemia in C3H mice fostered by high-leukemic AK mothers. These results, although not defining the mechanism responsible for the differences in reciprocal hybrids (male only), nevertheless did not produce evidence of a specific maternal factor capable of inducing leukemia. Kirschbaum and Strong (1942), using F strain mice, a strain having an incidence of leukemia of approximately 50%, of both lymphocytic and granulocytic forms, could find no evidence in reciprocal crosses of a maternal influence, though a slight reduction in the incidence of leukemia was noted in F strain mice fostered by low-leukemic mothers of the CBA, C57BL and A strains. In this case, as in the results of Furth *et al.*, the specificity of the foster-nursing influence was not determined.

A question of sex linkage naturally arises in interpreting the results of the differences observed using the high-leukemic AK strain (Cole and

Furth, 1941; Furth *et al.*, 1942), since these differences were confined to the males alone. This explanation seems unlikely since in another cross to be discussed later, between the AKR and NH strains (Law, 1953), there were obtained differences in reciprocal crosses in both sexes alike. Nor is this difference between sexes in the incidence of leukemia related

TABLE XI
Distribution, Incidence and Age at Death of Leukemic F_1 Females (♀ STOLI x ♂ C58) from Young and Old STOLI Mothers*

Length of Life (days)	Mothers†	
	Young	Old
300	1	—
350	4	—
400	5	—
450	5	—
500	8	—
550	9	2
600	10	1
650	7	5
700	4	6
750	4	4
800	1	4
850	4	7
900	—	13
950	—	3
1000	—	3
1050	—	1
1100	—	1
Total no mice	75	88
Per cent leukemic	82.6	56.8
Mean age at death, days	567	786
Difference 25.8 ± 2.7; P =	<0.001	

* Adapted from data of MacDowell and Taylor (1948).
† Average age of young mothers at parturition 14 weeks; of old mothers, 36 weeks.

to longevity since the data of Cole and Furth show that females die from causes other than leukemia at an earlier average age than males. Nevertheless this is a problem worthy of further investigation for it leaves some doubt as to the existence of a maternal influence in these particular crosses.

MacDowell and Taylor (1948) were able to show that the maternal influence responsible for the difference in incidence of leukemia in reciprocal F_1 hybrids between high-leukemic C58 and low-leukemic STOLI mice

consisted of a definite and specific resistance contributed by the low-leukemic mother. The resistance effect was not found at earliest sexual maturity but became increasingly potent with advancing age of the STOLI mother. This is shown in Table XI. Old mothers, 34 weeks and older at parturition, unquestionably reduced the incidence of leukemia and lengthened the age at death of leukemic mice, that is, leukemia appeared later in the group of F_1 hybrids (STOLI/C58) born of old mothers. Resistance was not evident in F_1 hybrids born of young STOLI

TABLE XII

Influence of Age of Resistant Mother and Nurses (STOLI) on Incidence of Leukemia and Age at Death on F_1 (STOLI x C58) Females*

	Mother-Nurse†											
	1 Young—Young			2 Young—Old			3 Old—Old			4 Old—Young		
	No.	%	Age at death	No.	%	Age at death	No.	%	Age at death	No.	%	Age at death
Leukemics	156	80.1	578.6	72	66.6	817.5	95	55.9	805.6	58	69.9	749.1
Nonleukemics	39	—	609.7	36	—	800.6	75	—	810	25	—	721.7

* Data adapted from MacDowell *et al.* (1951).

† *Average age of young mothers and young nurses 15 weeks; old mothers* and nurses average age was 36 weeks.

mothers, average age at parturition, 16 weeks. The incidence of leukemia in this group of F_1 hybrids (♀ STOLI x ♂ C58) was 82.6% and in the reciprocal F_1 hybrids (♀ C58 x ♂ STOLI) 85.3%. Thus, when resistance is absent, genetic influences show full dominance whether introduced by the C58 mother or father. Though it was found that there was a close association of less leukemia and longer lives, the nonleukemic mice with older STOLI mothers lived longer, indicating nonspecific factors delaying all causes of death.

Further evidence was presented that the maternal resistance factor (MRF) was transmissible by milk and also transmissible before birth, either as a cytoplasmic factor or transplacentally (MacDowell *et al.*, 1951). By reference to Table XII, column 2, it may be seen that F_1 hybrid offspring having young resistant (STOLI) mothers, but fostered-nursed by old resistant females show a lower incidence of leukemia and live longer than the control group born of young resistant mothers and nursed by them. The other control group, mice born of old resistant mothers and nursed by them (column 3), although having the same life span as the young-old group, show a still lower incidence of leukemia. The data of

column 4, mice born of old mothers but nursed by young STOLI females, indicated that the resistance influence, whatever the nature, is transmissible before birth.

The possibility should be tested whether the milk-transmissible resistance factor is detectable using the high-leukemic C58 strain. Preliminary evidence by MacDowell *et al.* (1951) indicates that it is. It is of interest also to determine if this resistance factor is specific for the leukemias arising in the C58 strain or is also capable of preventing the occurrence of spontaneous leukemia in other leukemic strains and of forms of leukemia other than lymphocytic. Through the use of the bioassay test designed by Furth and Boon (1945) or by employing transplantable leukemia, it should be possible to determine whether MRF exerts its influence directly on leukemic cells by suppressing cell division and growth or by modifying the conditions under which the transformation from normal to leukemic cell occurs.

It has been suggested that a neutralization phenomenon, similar to the Rh phenomenon, intensified during subsequent pregnancies in the resistant, STOLI, mother is operative (Furth, 1946b). This seems unlikely since it is not necessary to introduce C58 sperm to obtain the resistance effect. STOLI females which have produced only STOLI offspring are able to transmit MRF. Secondly, nursing alone seems to be quite as effective as prenatal environment plus nursing. Since the evidence shows that MRF, which may be contributed before birth or through milk alone, is not found at earliest sexual maturity in STOLI females but becomes increasingly potent with advancing age, the reciprocal differences shown in males only by Cole and Furth (1941) and Furth *et al.* (1942) and by Law (1952a), for both sexes (see Table X) cannot be related specifically to maternal resistance. The design of the experiments does not permit a determination of the influence of mothers' age. Furthermore, it seems unlikely that the differences reported by Furth *et al.* in crosses between the AK and C3H strains can be related to a specific resistance contributed by C3H mothers. A later cross by Law (1953), between the C58 strain and C3Hb, uncomplicated by the milk agent, failed to show differences in the reciprocal F_1 hybrids obtained (column 6, Table X).

It appears that the low-leukemic NH strain contributes a maternal resistance influence, similar to that of the STOLI strain, which increases with parturition age of the female, and which suppresses the development of leukemia equally in both sexes (Law, 1953). In contrast to MRF in STOLI females, a definite resistance is apparent in the NH strain at earliest sexual maturity (see Table XIII). Length of life of leukemic mice is strikingly increased and offspring born of NH mothers 40 weeks or older (not shown in table) show only 16% leukemia compared with the

53.3% leukemics born of young AKR mothers. Longer life of nonleukemic mice was also found to be associated with increase in parturition age of the low-leukemic (NH) mother. Thus, the association of longer life and less leukemia would appear to be not causal.

TABLE XIII

Influence of Mothers of Low-Leukemic NH Strain on Incidence of Leukemia and Age at Death of F_1 Hybrid Mice*

Cross	Age of Mother	No. of Mice	No. Leukemic	Per Cent Leukemics	Age at Death (months)	
					Leukemic	Nonleukemic
♀ H x ♂ L†	<32 weeks‡	77	40	53.3	16.5	15.5
♀ L x ♂ H	<32 weeks	98	36	35.9	18.4	18.5
♀ L x ♂ H	>32 weeks	42	11	26.2	21.9	19.7

* From data of Law (1953).

† H, high-leukemic AKR strain; L, low-leukemic NH strain.

‡ Average parturition age of mothers in first group is 15 weeks, in second group, 19.4 weeks and in third group, 36 weeks.

C. *Effects of Specific Genes.* Unmistakable evidence has been presented to show that susceptibility to leukemia in the mouse has a genetic basis. The pattern of inheritance appears similar to that described for mammary cancer and pulmonary tumors. Several genes appear to be involved and the expression of the character, leukemia, is determined by the combined effects of genetic and nongenetic factors. In the case of the C58 strain of mice, nongenetic variables account for the 10% nonleukemics developing in this genetically homogeneous population. Still another strong, nongenetic variable, the maternal resistance factor, accounts in certain instances for the nearly complete suppression of leukemia, despite the genetic tendencies of the animals.

At least two known genes of the mouse have been shown to be associated with susceptibility to leukemia. The association of the gene for dilution (*d*) and leukemia was shown by MacDowell *et al.* (1945). A similar association of the flexed-tail gene (*f*) and leukemia was shown by Law (1952a). This gene also produces a transitory siderocytic anemia in mice. It is indicated that both genes, *d* and *f*, which are plus modifiers for leukemia, themselves directly influence susceptibility. Both genes were introduced into the respective crosses from the low-leukemic strains, STOLI and WA. The flexed-tail gene has been shown to be associated with a decrease in susceptibility in pulmonary adenomas, and the linked genes, shaker-2 (*sh-2*) and waved-2 (*wa-2*), which also were found to be associated with a decrease in susceptibility to pulmonary tumors (Heston

and Deringer, 1947), have no relationship to leukemia (Law, 1952a). Thus specific genes appear to be associated with the development of specific neoplasms.

It appears likely that leukemia in the mouse is a genetic entity. Genes which determine susceptibility to leukemia may not be specific as regards the morphologic type of the disease. In various crosses, F_1, F_2, and backcrosses to the low-leukemic strain, where the number of susceptibility genes is reduced, it appears that other variables have an influence not only on the incidence of leukemia but on the induction of a specific cell type (Law, 1952a; Law, 1953). Though the predominant form of leukemia in the high-leukemic C58, AKR, and DBA strains is lymphocytic, in outcrosses to low-leukemic strains there are observed many other forms not encountered in the parent strains: granulocytic, reticulum-cell sarcoma, plasma cell leukemia, Hodgkins-like lesion, etc.

D. *Site of Gene Action.* Some progress has been made in a study of the physiologic genetics of mammary cancer and pulmonary tumors in the mouse. Paths of gene action in the development of mammary cancer, as outlined above, have been identified as existing (1) in the control of the propagation and transmission of the milk agent, (2) in the control of hormonal stimulation, and (3) in the control of the response of mammary tissue to these two stimuli. The primary action of the influential genes, relating susceptibility to pulmonary tumors, appears to be localized in the lung tissue itself. In the development of spontaneous lymphocytic leukemia in the mouse a complex situation is encountered which has not been adequately resolved. Clearly the basic change in leukemia resides in the leukemic cell itself and consists of failure to complete differentiation and the ability to overcome the forces which restrain the growth of normal immature cells. The change is strikingly neoplastic in character. Periodically, various ideas are revived which emphasize the basic change in leukemia as occurring in the host rather than in the cell. Ziegler's "equilibrium disturbance" (Miller and Turner, 1943; Foster and Miller, 1950), the disturbance of normal elimination of leukocytes (Bierman *et al.*, 1951), the various infective agents said to be associated etiologically with Hodgkins disease (Gordon, 1936; Lundback and Løfgren, 1950), or the idea that leukemia is a "deficiency state" are interesting phenomena, the biologic significance of which is important, but there is no evidence of their relationship to the etiology of leukemia. The simple fact remains that all the various manifestations of leukemia result following transfer, to an appropriate host, of a single leukemic cell (Furth and Kahn, 1937). Furthermore, as shown by Lewis (1937), deBruyn *et al.* (1949), and others, leukemic cells multiply *in vivo* and retain their distinguishing characteristic features.

An attempt has been made to arrive at some understanding of the basic change involved in the transformation from the normal to the leukemic cell (Law, 1952b). In the high-leukemic AKR strain of mice the thymus is initially and principally involved in the leukemic process. Of 62 leukemic mice (all lymphocytic leukemia) of this strain, 60 or 96.8% showed moderate to severe thymic involvement. In many cases the thymus was the only tissue found to be leukemic. Upon transplantation of these spontaneous leukemias into AKR mice or into F_1 hybrid mice having AKR as one parent, a typical systemic leukemia results which is then transplantable back to the AKR strain. That thymic tissue, through some mechanism, influences the induction of leukemia has been shown by Furth and his associates (McEndy *et al.*, 1944; Furth, 1946a) for the AK strain, by Law and Miller (1950a,b) for the C58, AKR, and DBA strains and by Kaplan (1950) for the C57BL strain of mice. Total thymectomy in these strains strikingly decreases the incidence of leukemia and increases the average length of life of those mice which become leukemic. In the DBA strain, though total thymectomy reduced the incidence of carcinogen-induced leukemia from 69.7 to 22.0%, thymectomy followed by immediate autoplastic grafting of the thymus into the subcutaneous connective tissues resulted in an incidence of leukemia similar to that of the intact controls, 69.1%, indicating that the presence of thymic tissue in the body is a necessary antecedent to the development of leukemia in a high percentage of mice of this strain. Furth and associates interpret their findings to mean that the thymus contains "potentially malignant" cells that upon removal reduces the probability of the development of leukemia. On the other hand, evidence obtained by Law and Miller (1950a,b), Kaplan (1949, 1951), and Lorenz (1950) indicates a more indirect role of the thymus.

With this background of experimental data an experiment was designed in an effort to elucidate the possible role of the thymus in the development of leukemia in AKR mice. Fragments of thymic tissue, from 4-week-old AKR mice were transplanted to F_1 hybrid mice produced by crossing low-leukemic C3Hb females and high-leukemic AKR males. Normal tissues would be expected to persist and grow in such mice. A statistically significant increase in the incidence of lymphocytic leukemia was found in the (C3Hb x AKR) F_1 mice bearing transplanted fragments of thymic tissue from the high leukemic AKR mice. Transplants of thymic tissue were recovered in 26 of the 28 leukemic F_1 mice and all except 2 were found to be leukemic. The recovered thymic tissues grew progressively in F_1 hybrid mice, producing generalized leukemia but, contrary to expectations, failed to grow progressively and produce leukemia in AKR mice, the strain of origin of the tissues. They behaved in

transplant like the F_1 host leukemic tissues. More recent unpublished work shows that homogenates or filtrates of AKR thymuses failed to increase the incidence of leukemia over control mice. It was observed in the above experiment that the transplanted thymic tissue underwent degenerative changes in which the thymic round cells disappeared followed by a reappearance of round cells and full reconstitution of thymic tissue. Reticular elements remained unchanged. It is apparent from these results that the round cells in the reconstituted thymic tissue have infiltrated from the F_1 host and in due time have become transformed into leukemic round cells. The mechanism of transformation through which genetic influences act is still not clear. Thymic tissue in some manner influences the transformation, but a simple explanation, such as that available for the induction of pulmonary tumors, is not indicated at present.

E. *Exogenous and Endogenous Agents in Causation of Leukemia.* The basic change from normal to leukemic, which is intrinsic to the cell, may be the result, first, of an alteration of the reproductive material of the cell. The specific genes which have been identified as influencing susceptibility to leukemia could conceivably do so by producing gene mutations in the body cell. The somatic mutation theory maintains that a cancer is a consequence of the mutation of a gene in a body cell. Since one of the diagnostic properties of a gene is that it is self-reproducing in either the original or mutant form, the effect of mutation would be maintained and reproduced. The basic change, secondly, may be the result of the presence of a self-perpetuating agent of exogenous origin, a virus or virus-like agent. Gross (1953) has revived the virus etiology concept of leukemia and this will be discussed in detail. Thirdly, a mutant factor of endogenous origin (plamagene) in the cytoplasm, which is reproducible and which causes abnormal differentiation, may be the etiologic agent.

The many other chemical and physical agents which influence the expressivity of leukemia in inbred strains of mice, estrogens, x-rays, carcinogens, nutritional factors, etc., are considered to be modifying factors. It is likely, for example, that carcinogenic agents which are also known to be mutagens (Demerec, 1947, 1948; Auerbach, 1949) produce gene mutations in the body cells or plasmagenes or make the cells more receptive to agents of exogenous origin.

Experiments relating to the third possibility discussed above have been reported recently by Stasney *et al.* (1950). These authors reported the successful transmission of the Murphy rat lymphosarcoma by inoculation of chromatin fractions isolated from lymphosarcomatous cells. They noted the development of lymphosarcomas at the site of transplantation followed by the development of a generalized leukemia. It was contended

that altered "chromatin" particles, or some component, obtained from leukemic cells brought about a neoplastic transformation of the lymphocytes in the recipient animals, presumably at sites of lymphocytic cells in the subcutaneous tissues. This is unlike the leukemias of the fowl, where induction of leukemia is at a common site of lymphoid tissue. The critical problem in these experiments has been resolved in a subjective manner. Since intact leukemic cells have never been observed in any of their centrifugated preparations, it was assumed by these investigators that the neoplasms arose *de novo*, that is were induced, and did not result from contamination of their preparation with viable cells. By the use of a simple genetic test it is possible to determine, in any given situation using homogeneous strains of animals, whether tumors developing after the inoculation of tumor preparations really represent a *de novo* induction of a tumor in the host tissue or are tumors arising as the result of transfer of viable cells. It was shown by Law (1951), using mice of different genetic constitutions and mammary carcinomas with and without the milk agent, that the interpretations of Mann (1949a,b), suggesting an "activation" of the milk agent, following freezing or freeze-drying, were erroneous. Living tumor cells were found to be transferred in similar procedures, using controlled tests. Recently Klein (1952), using this objective genetic test, was able to show that lymphomas did develop after the subcutaneous inoculation of chromatin fractions, as shown by Stasney *et al.*, but that the growths resulted from the transfer of viable cells contained in the chromatin fractions.

Whereas Stasney and associates consider the etiologic agent of leukemia to be a self-reproducing particle of the cell, Gross conceives an agent transmitting leukemia to be an infectious virus. In his original report, Gross (1951a) describes the induction of lymphocytic leukemia in mice of the C3H strain following inoculation, within 12 hours post partum, of centrifugated extracts of leukemic cells or normal embryos of the AK high-leukemic strain. Similar inoculations were said to be unsuccessful if the recipient C3H mice were older than 12 hours. It was concluded that the spontaneous leukemia of AK mice is caused by a filterable agent similar to that of avian lymphomatosis. This early work was commented upon, critically, by Furth (1951). Later (Gross, 1951b, 1952) published additional evidence to support his hypothesis of the viral etiology of leukemia. In view of the importance of this work it deserves a critical appraisal. More recent findings reported by Gross are these: (1) recipient, low-leukemic C3H mice need not be less than 12 hours old to become leukemic, following inoculation of AK leukemic cell centrifugates. Test mice as old as 8 days become leukemic in a small number of cases, but considerably later than younger mice, when given inoculations of leukemic

cell centrifugates or of filtrates. (2) Filtrates prepared by passing the centrifugate through a Seitz (S-1) filter induced leukemia in a small percentage of C3H mice. (3) Transmission of the "agent" was indicated in first-generation off-spring obtained by mating *inter se* C3H mice which had obtained centrifugate or filtrate preparations.

It is not clear from the description of these experiments whether appropriate control procedures were followed. Since littermate controls were not used, the question arises as to whether the leukemias which appeared in the test mice were the direct result of the inoculations. It is known that spontaneous leukemia in the C3H strain is indeed rare (Gardner *et al.*, 1944; Furth *et al.*, 1942; Law, 1952a), but this fact alone does not constitute a control of an experiment. The source of the C3H mice used by Gross is given, but again one should inquire if these mice were maintained as pedigreed mice in brother x sister matings, to rule out the possibility of contamination. It is known, and has been shown by Gross (1950) for the strains of mice used in his investigations, that leukemic cells (AK) will grow progressively, causing a generalized leukemia and death within 2 to 3 weeks, when inoculations are made into newborn mice of the C3H strain. The older the recipient mice, the fewer the number of progressively growing "takes"; although even in mice of weaning age, 16 to 30 days, nearly 20% progressively growing tumors were obtained following inoculation of AK leukemic cells into mice of the C3H strain. This is expected, since strains AK and C3H have the same histocompatibility allele in common ($H\text{-}2^k$) (see section on Transplantable tumors). Some years ago Furth *et al.* (1944) pointed out that AK leukemic cells could be grafted on C3H strain adult mice and grow progressively, and we have regularly transferred leukemic cells from AKR mice to the C3H strain with resultant leukemia (Law and Boyle, 1953). Thus it may be unfortunate that these two strains of mice were chosen by Gross for his investigations.

Centrifugation of leukemic cell preparations at 3000 rpm for 10 minutes as done by Gross does not guarantee a cell-free material. It is known, especially with leukemic growths, that when the number of cells in the inoculum is small the transferred cells may lie dormant, or at least give no evidence of progressive growth, for months. This is the likely explanation for the delayed effects of inoculation of AK leukemic cells in mice of the C3H strain, as reported later by Gross (1952). His explanation of an "inactive" virus, nonpathogenic for the carrier host, but which is activated in middle age, does not seem plausible. Such an hypothesis may be tested by the simple genetic test, described earlier (Law, 1951; Klein, 1952). In an effort to determine the nature of the leukemia which developed in C3H hosts, two of the leukemias were transferred to C3H

hosts and grew progressively in a total of 8 mice (Gross, 1951). The original AK leukemias, however, were not transplanted into C3H mice to provide control material.

Though Furth (1951), in a critical review, argued that the results of Gross cannot be reconciled with the known genetic facts of leukemia, it should be pointed out that the milk agent, a virus-like agent, is under the control of genetic influences. Nonetheless, if a virus etiology of leukemia is accepted, the sperm as well as the ovum is capable of transmitting the virus, as may be inferred from the results of reciprocal crosses, and it is not transferred by way of the mother's milk (MacDowell and Richter, 1935; Furth *et al.*, 1942). Unpublished work by Fekete (1953), which may have some bearing on this problem shows that AKR mice, obtained by transplanting AKR fertilized ova to C3H strain females, develop leukemia at approximately the same rate as the AKR strain. A critical test is the reciprocal transfer of C3H fertilized ova to AKR females.

The objections raised to the work of Gross are of a serious nature and should be considered in attempts to reproduce these results.* Many investigators (Richter and MacDowell, 1933; Furth and Kahn, 1937; Rask-Nielsen, 1938; etc.) have been unsuccessful in transmitting leukemia by cell-free extracts. The pitfalls in such investigations are many, as evidenced by earlier reports of the viral etiology of leukemia (Engelbreth-Holm and Frederiksen, 1938) which have not been confirmed. Confirmatory data obtained under the most rigidly controlled conditions will be required to establish the reality of the effects noted. Though agents with virus-like properties are known to be associated with certain lines of transplantable leukemia, modifying the expression of the disease (Taylor and MacDowell, 1949; de Bruyn, 1949; Law and Dunn, 1951), these appear in no manner to be associated with the etiology of leukemia.

The possibility of transduction of tumors by cell-free extracts, as indicated in the work of Stasney *et al.* (1950) and by Lettré (1950) must be considered as a likely phenomenon in view of the rather general character of such transformations in bacteria (Lederberg, 1952). Such phenomena will be found and elucidated only in a controlled genetic

* More stringent controls have been used by Gross (1953) in extensions of this work. It has been observed that littermate control C3H mice inoculated with heated centrifugate preparations (7000 x g for 10–15 minutes) failed to develop leukemia, whereas those given unheated centrifugate have developed to date 44% leukemia at 5.4 months. This evidence is suggestive, but not conclusive, that cell-free material is inducing leukemia in the C3H test mice, since the additional information has been obtained that the leukemia so produced is transplantable to adult mice of the C3H strain but not to AK mice, original source of the leukemic tissues.

situation, and it is predicted that the pathologic consequences and propagation of any transducing agents will be strictly under genic control.

4. Other Neoplasms

There are a number of different types of tumor reported as occurring predominantly in certain inbred strains of mice. A hyperplastic lesion of the stomach of I strain mice has been described (Andervont and Stewart, 1937; Stewart and Andervont, 1938). The principal lesion which occurs in the pyloric chamber of the glandular portion of the stomach appears not to be malignant. The regular occurrence in this strain clearly suggests a genetic origin of the anomaly but critical genetic studies have not been done. It is known however, that the lesion does not appear in F_1 hybrids from out-crosses to the C57BL strain, and data obtained from back-crosses indicate that several genes may be involved. Strong (1945) reported a subline of mice developed by selection and routine administration of a carcinogen, methylcholanthrene, in which a high incidence of gastric carcinoma occurs. This may prove to be a highly important strain if confirmation is obtained of the diagnosis of adenocarcinoma, distinct from the adenomatous lesion which occurs commonly. This seems not to have been done to date. That the sex difference in incidence of gastric lesions in this subline is influenced by sex hormones and is not genetic in character has been shown by the work of Smith and Strong (1949).

Hepatomas occur fairly frequently in the CBA strain (Strong and Smith, 1936; Gorer, 1940) and in the C3H strain (Andervont, 1939). These two strains trace back to a common source (Heston, 1949a). Though this condition was thought to be benign, recent work by Andervont and Dunn (1952) indicates the probable malignant nature of spontaneous and induced hepatomas. Marked interstrain differences have been noted in the induction of hepatomas by various carcinogens: carbon tetrachloride (Edwards, 1941; Edwards *et al.*, 1942a,b), 2-acetylaminofluorene (Armstrong and Bonser, 1947) and by several azo dyes (Kirby, 1945). It is presumed but not shown that these strain differences are genetic.

Subcutaneous neoplasms, mostly fibrosarcomas, are induced easily by the subcutaneous injection of the carcinogenic hydrocarbons. Strain differences have been shown to exist (Andervont, 1938a,b, 1940a); these again are presumed to be genetic. In crosses between various strains, differing in susceptibility the first generation hybrids were found to be intermediate to the parent strains. The recent results of Burdette (1943) are similar to those reported by Andervont. No evidence of an extra-chromosomal influence or of sex linkage was found in either study.

An influence of age of mothers affecting several characteristics of

induced fibrosarcomas has been reported by Strong (1948a, 1950a,b,c, 1951). A subtle and as yet undefined mechanism, which changes during the lifetime of the mother and is passed on to the progeny in successive litters, was described in a subline of mice whose ancestors were selected for resistance to chemically induced tumors: (1) The rate at which fibrosarcomas appear at the site of injection of methylcholanthrene was found to be influenced by litter sequence; susceptibility was lowest in mice of the first and second litters and highest in the fifth and sixth litters; (2) The survival time of mice with induced fibrosarcomas increased with the litter frequency of the mother. Mice born in a first litter lived only 52.4 days. This survival time was found to increase gradually to the final litter recorded, the eighth: average survival of a mouse belonging to an eighth litter was 130.0 days. (3) An increasing sex differential was also found, increasing with successive litters. (4) Finally, the percentage of mice showing invasive tumors gradually diminished in mice of successive litters. Three other lines of descent, some related, show similar maternal influences. The interrelationship of the observed effects and the role genetic factors play in the described mechanism remain to be determined. Strong (1948a) considers the mechanism discussed as a "resistant" mechanism highest in young females and diminishing in effectiveness with advancing age. If so, this is in striking contrast to the resistance mechanism, discussed earlier, which influences susceptibility to leukemia.

Extensive experiments involving the use of the powerful carcinogen, methylcholanthrene, have been described by Strong (see 1945 and 1948b). In order to create a high degree of genetic variability, Strong made crosses involving three established inbred lines of mice, CBA, JK, and N. The resulting NH stock showed a low incidence of spontaneous neoplasms. Beginning with the fourth generation of inbreeding of the NH stock, all breeding mice were then injected with 1 mg. of methylcholanthrene at 60 days of age. Selection was instituted of those individuals which best resisted the development of subcutaneous tumors. The consequence of this procedure was that many animals survived to develop tumors at sites remote from the injection. A multiplicity of tumors in many sites appeared in this heterogeneous stock. In addition many visible mutations made their appearance some of which could be shown to be heritable. Of particular importance it was reported, as discussed earlier, that gastric carcinoma appeared in the injected (NHO) line. Subsequently, gastric carcinoma continued to appear in a line in which treatment with the carcinogen was discontinued. Strong proposed that the action of the carcinogen was twofold: (1) a germinal gene mutation for susceptibility to gastric carcinoma was induced, and (2) a transformation (somatic mutation) to cancer occurred in the gastric mucosa. It is to be remem-

bered that a genetically heterogeneous stock was employed in this study. Selection of resistant mice following the use of a carcinogen precluded the maintenance, simultaneously, of control mice. Hence, there appears no way of determining whether the numerous variants observed by Strong came into being as a result of selection, probably fortuitous, or as a result of the occurrence of mutations, induced or otherwise.

Very little is known about the physiologic mechanisms which make one strain more susceptible to the induction of a tumor than another. Do the physical and chemical carcinogens act as accelerators or do they, in certain cases at least, initiate a completely different physiological process leading to malignancy? As a rule, a neoplasm which occurs spontaneously in an inbred strain may also be obtained more quickly and usually in a higher percentage of animals than neoplasms which occur rarely. Pulmonary adenoma are elicited readily in susceptible strains by various chemical and physical agents, and it appears very likely that this is an acceleration of the physiologic mechanism which normally leads to pulmonary adenoma formation, since specific marked genes are implicated in each (spontaneous or induced) neoplasm. In genetically susceptible mammary-cancer strains, carcinogens strikingly increase the incidence of the lesion. This has been shown, for example, by Andervont and Dunn (1950b) in a milk agent-free strain. In the same strain of mice, DBA, an earlier appearance of mammary cancer occurs following administration of the carcinogen if the milk agent is present (Engelbreth-Holm, 1940). On the other hand mammary cancer appears rarely in carcinogen-treated resistant strain mice (Dmochowski and Orr, 1949). The milk agent-free C3H strain (C3Hb) is probably one of the most susceptible to spontaneous fibrosarcomas of the subcutaneous connective tissue (Heston, 1951). An increase in the incidence of sarcoma has been observed in irradiated C3Hb females (Lorenz *et al.*, 1951). On the other hand, an apparent exception to the rule is observed in leukemia. Though it has been shown that carcinogens and x-rays induce leukemia readily in specific strains of mice (DBA, A and C57BL), a relationship between genetic susceptibility and induction of the neoplasm is not clear nor is there clear evidence of additive effects of the various carcinogens. The considerable response elicited by X-ray particularly in the C57BL and A strains, in which leukemia is not characteristic is what one might expect however, if the development of a tumor is a threshold phenomenon, expression of which results when the combination of genetic and non-genetic factors surpasses the threshold. Some low-tumor strains are far below the threshold and in such cases response to a carcinogen may be small. In contrast certain low-tumor strains exist which are only slightly below the critical threshold; the carcinogen may thus lift it above the critical level and pro-

duce a striking effect. The possibility exists that carcinogens in certain cases institute an entirely different physiologic process leading to malignancy, in which case an additive effect of genetic and non-genetic mechanisms would not appear. Until the precise mechanisms are known determining susceptibility and resistance to neoplasms, the hope of distinguishing these possibilities appears remote.

5. Remarks on Inheritance of Cancer

The facts related in this review of genetic studies in experimental cancer definitely exclude any simple genetic interpretation. Many genes appear to be involved determining susceptibility to each of the neoplasms investigated. These genes are cumulative in effect. The inheritance of the characters described, pulmonary adenomas, mammary cancer, and leukemia resemble closely the inheritance of such characters as polydactylism in the guinea pig (Wright, 1934) and skeletal defects in the mouse (Grüneberg, 1952b). The expression of the character depends upon the segregation of multiple genes in conjunction with a threshold of manifestation. Certain stigmata characteristic of this type of inheritance (Grüneberg, 1952b) are quite obvious in the genetic studies of cancer. The character is generally sensitive to influences of the environment, either intra-uterine or acting later in life. The tendency for different expressions of the neoplasm in reciprocal crosses as a result of permanent differences in maternal physiology is quite evident particularly in the study of leukemia. The expression of cancer is also extremely sensitive, in certain cases, to maternal age or parity which represent progressive changes in the maternal physiology. The differences in expressivity of fibrosarcomas, of leukemia, and of mammary cancer seem directly related to changes in the maternal physiology. General genetic effects like sex which influence the expression of several skeletal defects in the mouse are also known to influence the expression of hepatomas, gastric lesions, and leukemia. Pulmonary adenomas, on the other hand, appear less sensitive to any of these influences. The sensitivity of various neoplasms to the influences of environment has erroneously led to the impression that the nongenetic variables are all important. It should be recognized that only in a critical genetic analysis is it possible to evaluate any environmental effect.

Much more attention should be given in future genetic investigations to such maternal effects which have been described in this review: intrauterine effects, influences of age of the mother, and/or parity, etc. Little is known, for example, of the specificity of these effects, and the design of some experiments precludes the detection of them. A fundamental difference is to be noted between "maternal inheritance" or "maternal transmission" and the maternal effects which have been described and appear

transitory in nature. In the former, self-reproducing entities are handed on to the young by the mother, either as a normal constituent of the cytoplasm, of which no example is known in mammals, or through a virus-like infection, for example the milk agent.

It is the opinion of Grüneberg that the multiple genes which are responsible for the expression of diverse skeletal defects in the mouse are in fact genes which have other primary effects but whose remote effects only are being studied. This would explain their sensitivity to environmental influences. There is some evidence that this is true in cancer; a direct relationship between several genes and susceptibility has been shown.

The neoplasms discussed at greatest extent in this review appear to fit into a common pattern of inheritance. There is no reason for assuming that future genetic studies will not bring to light neoplasms which are entirely dependent upon genetic factors for expression or those which, on the other hand, are to a great extent influenced by environmental factors.

III. Genetics of Tumor Transplantation

The argument that transplantable tumors contribute little to an understanding of the etiology of cancer is quite sterile and emphasizes a lack of appreciation for an immense amount of literature contributing basic information to the field of oncology. Studies with transplantable tumors have given information concerning the autonomy of malignant growths and concerning their histogenesis. Basic facts concerning tumor immunity are now being gradually uncovered. With the development of proper, genetically developed sublines of mice, so-called isogenic resistant sublines, discussed in detail later, it will be possible to detect, analyze, and characterize somatic mutations in cancerous tissues. The possibility of detecting somatic segregation in tumor cells, originating for example in heterozygous animals, should not be overlooked.

The use of heterogeneous mice in the study of transplantable tumors gave rise to unpredictable results and many theories to account for these results. The periodic changes, "rhythms" (Bashford *et al.*, 1908) in tumors, thought to result from inherent changes in the tumor cells, are known to be artifacts (Strong, 1926c), and such variations have been reproduced by the use of controlled genetic material (Bittner, 1932).

The impetus to studies of transplantable tumors in mice of known ancestry was given by Leo Loeb (1902), who observed that a tumor which arose spontaneously in a mouse of the Japanese waltzing strain grew progressively in all mice of this strain but failed to grow in ordinary white laboratory mice. These findings were verified by Tyzzer (1909) and by Tyzzer and Little (1916) using an adenocarcinoma of the mammary

gland. This tumor grew in 142 of 145 Japanese waltzing mice, in 69 of 70 F_1 mice, but, paradoxically, failed to grow progressively in 54 F_2 and 16 F_3 mice. It was shown subsequently (Little and Tyzzer, 1916) using a larger series of mice, that some of the F_2 mice (3 out of 183) did grow the tumor following transplantation, thus showing that transplantability which behaved like a dominant character in the F_1 did not escape detection in the F_2 generation. Little (1914) first suggested, from the above results, that susceptibility to the transplanted adenocarcinoma depended on the simultaneous presence of dominant genes derived from the strain (Japanese waltzer) in which the tumor arose. Thus, the parent strain would carry all these genes, in homozygous condition; the genes were carried in the F_1 in a heterozygous condition, giving rise to 100% susceptibility. Only those F_2 mice would grow the tumor progressively which carried all the dominant genes simultaneously. In this particular cross it was estimated that 12 to 14 independent dominant genes were concerned with tumor susceptibility. This was estimated from the formula $f = (\frac{3}{4})^n$ where f is the fraction of animals growing the tumor progressively, and n is the number of dominant genes involved. It is to be noted however, that since neither strain used was highly inbred this estimate was indeed unreliable because of the large sampling error.

The genetic theory of tumor transplantation advanced by Little was extended by Little and Strong (1924) and consolidated and confirmed by many investigators. Although most of the early work was accomplished with mammary adenocarcinomas, especially in the DBA and A strains, other investigators have shown that the concept is of general applicability by the use of various other inbred strains and other morphologic types of tumors, ovarian (Strong *et al.*, 1938), leukemia (MacDowell and Richter, 1930a, 1932), sarcoma (Furth *et al.*, 1944; Gorer, 1937), etc.

Essentially, the facts concerning transplantability of tumors are these: When a tumor is transplanted into inbred strains of mice and into the various hybrid generations it is found that (1) the tumor grows progressively and kills in all animals of the strain, or more specifically the subline, of origin, (2) it fails to grow progressively in unrelated strains, (3) it grows in all F_1 animals where one parent is from the strain of origin of the tumor, and (4) it grows in a certain proportion of F_2 mice and backcross mice (F_1 x resistant parent), the proportion varying with different inbred strains and tumors. These results may be explained by assuming that a tumor will grow progressively and kill only in mice having specific dominant (or semidominant) genes present in the strain of origin of the tumor. There are certain modifications to the above laws which have been brought to light by recent genetic studies, and these will be discussed in detail later.

In a recent theoretical treatment of the genetic basis of tumor transplantation, Snell (1948) has proposed that the genes concerned be called "histocompatibility" genes and be denoted by the symbols *H* and *h* and, where specific genes are identified, by *H1*, *H2* . . . *Hn*. Thus, they may be defined as genes whose presence is required to ensure compatibility between tumor and host animal.

From the ratio of animals progressively growing a tumor to those regressing the tumor in the F_2 and backcross (to resistant strain) generations, estimates of the number of genes involved in tumor regression may be obtained. In the F_2 and backcross generations a certain proportion will grow a tumor progressively. This proportion does not exceed, within sampling error, 0.75 for the F_2 and 0.5 for the backcross. It is significant that in nearly all cases studied these 2 generations have given the same estimate of the number of genes involved.

Typical examples of results obtained in tumor transplantation, using inbred strains of mice, the F_1, F_2 and backcross (BC) generations are given in Table XIV. The inbred strains of mice used were the A and DBA

TABLE XIV

Transplantation Characteristics of Six Different Mammary Carcinomas in A and DBA Strains and in F_1, F_2 and Backcross Generations*

Case	Tumor	Strain of Origin	Strain of Mouse and Crosses between Strains										Probable No. of Histocompatibility Genes
			A		DBA		F_1		F_2		BC		
			+	−	+	−	+	−	+	−	+	−	
1	17495a	A	211	0	1	93	117	0	179	65	63	86	1
2	13738a	A	213	0	0	96	106	0	150	144	52	153	2
3	14905a	A	206	0	0	174	145	0	67	154	6	81	4
4	dbr D	DBA	0	104	94	0	125	0	16	96	—	—	7
5	16189a	A	137	4	9	8	38	0	4	110	0	101	12
6	19308A	(DBA x A) F_1	0	249	0	221	496	0	143	1434	28 / 131	874† / 799	8

* Adapted from data of Cloudman (1932), Strong (1926a), and Bittner (1933).

† These are figures for backcross to the A strain and backcross to the DBA strain. F_2 data give an estimate of 8 histocompatibility genes, the A backcross, 5 and the DBA backcross 3 *H*-genes.

strains. The transplantibility of adenocarcinomas of the mammary gland arising spontaneously in these 2 strains and in an F_1 hybrid mouse obtained by mating the A and DBA strains (case 6) is shown.

Many other experiments using inbred mice of various strains and numerous histologic types of tumor have given similar results except that the proportion of mice progressively growing the tumors in the F_2 gen-

eration and in the backcross generation, produced by crossing the F_1 to the resistant parent, have varied according to the tumor and strains used and the time during the course of transplantation when transplantability was tested.

1. In considering case 1 of Table XIV, adenocarcinoma 17495a, originating in an A strain mouse and tested for transplantability in the F_1, F_2, and DBA backcross generations, it may be seen that the tumor will grow progressively (+) only if a dominant histocompatibility gene (*H1*) is present either in the homozygous state (*H1H1*) or heterozygously (*H1h1*):

Strain or Cross	Genotype
A x DBA	*H1H1* x *h1h1*
	+ −
	(100%)
F_1	*H1h1*
	+
	(100%)
F_2	3 *H1h1*:1 *h1h1*
	+ −
	(75%)
F_1 x DBA	1 *H1h1*:1 *h1h1*
	+ −
	(50%)

2. Similarly, if case 2, Table XIV, is considered, it may be seen that adenocarcinoma 13738a, which arose spontaneously in an A strain mouse requires the presence, simultaneously, of 2 histocompatibility genes *H1H2* for progressive growth in the various hybrid generations:

Strain or Cross	Genotype
A x DBA	*H1H1 H2H2* x *h1h1 h2h2*
	+ −
	(100%)
F_1	*H1h1 H2h2*
	+
	(100%)
F_2	9 *H1h1 H2h2*:3 *H1h1 h2h2*:3 *h1h1 H2h2*:1 *h1h1 h2h2*
	+ −
	(56.25%)
F_1 x DBA	1 *H1h1 H2h2*:1 *H1h1 h2h2*:1 *h1h1 H2h2*:1 *h1h1 h2h2*
	+ −
	(25%)

In considering cases 3, 4, and 5 of Table XIV, it may be seen that the number (n) of dominant histocompatibility genes involved may be estimated by the formulas:

$(\frac{3}{4})^n$ for the F_2 generation, and

$(\frac{1}{2})^n$ for the backcross of the F_1 to the resistant parent.

3. Case 3, adenocarcinoma 14905a, gives excellent agreement between progressive growths obtained and expected results if it is assumed that the A strain carries 4 dominant histocompatibility genes which are necessary in the offspring of the various hybrid crosses for growth of this tumor. Thus, the A strain genotype is *H1H1 H2H2 H3H3 H4H4* and the DBA strain *h1h1 h2h2 h3h3 h4h4*.

4. Adenocarcinoma 19308A, case 6, arose spontaneously in an F_1 hybrid animal produced by crossing the DBA and A strains. This tumor failed completely to grow progressively in either parent strain but grew in all 496 F_1 mice. The results obtained in the F_2 and the backcross generations, to the A and to the DBA strains indicate that 8 histocompatibility genes were required for progressive growth of which 3 genes were contributed by the A strain and 5 by the DBA strain.

Data obtained from cases 3 and 6 of Table XIV are given in Table XV

TABLE XV

Observed Progressive Growth (+) of Transplants Compared with Expected Progressive Growth on Basis of Genetic Laws of Tumor Transplantation*

	F_2		ABC		DBA BC	
Tumor (strain)	+	−	+	−	+	−
14905a (A strain)						
Observed	67	154	91	0	6	81
Expected (4 genes)	70	151	91	0	5	82
Dev./S.E.	0.4				0.3	
19308A (F_1 DBA x A)						
Observed	143	1434	28	874	131	799
Expected (8 genes)	158	1419	28	874	116	814
Dev./S.E.	1.2		0.0		1.5	

* Adapted from data of Cloudman (1932) and Bittner 1933).

in comparison with the expected results assuming 4 and 8 genes respectively for these tumors.

Results obtained with tumors arising in animals of the F_2 hybrid generation have been meager but nevertheless consistent. Bittner (1935), using tumor ZNF_2 883, which arose in a cross of the C3H and N strains, found that no progressive growths were obtained in the one parental strain tested (C3H), all (128) F_1 hybrids grew the tumor progressively and a certain proportion of backcross mice were susceptible, compatible with the assumption of 5 to 6 histocompatibility genes necessary for progressive growth of the tumor. Furth and Barnes (1941) and Schweitzer and Furth (1939) obtained similar results.

From the foregoing discussion on tumors arising in F_1 and F_2 hybrids, two less important genetic laws may be added to the four given previously:

A tumor arising in an F_1 hybrid will grow progressively in all F_1 hybrids (identical to the F_1 in which the tumor originated) and will grow in a proportion of F_2 and backcross mice depending upon the tumor and the inbred strains used. It will not as a rule grow progressively in either parental strain.

A tumor arising in an F_2 animal will grow in all F_1 mice and a certain proportion of F_2 and backcross mice. It is possible that certain selected F_2 tumors will grow in all mice of one or the other of the parental strains.

The validity of the interpretations given: the relationship of genes to susceptibility and resistance of various transplantable tumors, although fitting the facts and being supported by standard errors, may be questioned. As pointed out by MacDowell these interpretations are based upon an all-or-none (progressive growth or no growth) classification of a probably complex phenomenon. Crucial evidence was lacking that the ratios obtained actually represented genetic differences. MacDowell and Richter (1932), however, have shown that susceptibility to a transplantable acute lymphocytic leukemia (line I), in C58 strain mice, depends upon a single dominant autosomal gene. The conventional method of breeding was used to show that a 1:1 ratio was obtained among backcross mice, using the STOLI strain as the resistant strain. A crucial second backcross (progeny test) was then made in which the proportion of offspring in each of 50 separate families was determined by inoculation. Twenty-five of these families gave ratios evenly distributed about the 50% level; thus, the backcross males were heterozygous for the gene in question; while 25 of the families were almost entirely resistant, lacking the gene. After the backcross males were mated to obtain the 50 families, they were then inoculated with line I leukemia. Of the 25 males whose offspring proved 50% susceptible, 17 grew progressively line I leukemic cells. These animals were much older at inoculation than those used as standard thus accounting for some of the negatives. None of the other 25 backcross males grew the leukemic cells progressively. However, 5 of the families gave 1 or 2 individuals that proved susceptible to the inoculations. Thus, it appears that susceptibility is influenced probably only to a minor extent by other as yet unidentified genes.

1. Transformations in Transplantable Tumors

It is a common characteristic of most all types of transplantable tumors that they change either morphologically or physiologically during the course of successive transfers. Physiologic changes involve a more

rapid growth with a shorter interval before death and, in some cases but by no means generally, an increase in the number of metastases. It is known, from several studies, that sudden changes or "mutations" occur such that the tumor requires a smaller number of histocompatibility genes for growth than it did previously. This is determined by testing in the F_2 and resistant backcross generations. It is assumed, but not proved, that increase in virulence and loss of specificity are the result of the same mechanism. Two instances of loss in specificity and histocompatibility gene requirements are given in Table XVI. Mammary adenocarcinoma

TABLE XVI

Transplantability of Original and Mutant Sublines of Tumor 17495a (A Strain) and Tumor dbr D (DBA Strain) in Test Mice of F_2 Generation*

Tumor	Generation	Progressive Growth	No Growth	Probable No. of Histocompatibility Genes
		17495a in A strain		
Original	F_1	117	0	—
Original	F_2	70	58	2
"mutant"	F_2	179	65	1†
		dbr D in DBA strain		
dbr D	F_1	125	0	—
dbr D	F_2	16	96	7
dbr DM	F_2	56	43	2
dbr DBL	F_2	66	56	2
dbr DBS	F_2	88	36	1

* From data of Cloudman (1932) and Strong (1926b).

† Backcross data gave identical *H*-gene requirements in this particular tumor.

17495a, an A strain tumor of Cloudman (1932) which originally required 2 histocompatibility genes for growth was found to have mutated so that it gave a one-gene ratio when tested at a later date. Three changes in loss of histocompatibility gene requirements have been noted by Strong (1926) within the course of transfer of the dbr D mammary adenocarcinoma in the DBA strain. As determined in crosses with the A strain, this tumor, when originally tested in the F_2 generation, required 7 genes for growth. Three sublines were developed from the original tumor and retested in F_2 individuals. One of the sublines, dbr DM, showed an increased proliferative vigor. It was found that this subline required only 2 genes for growths as did another subline dbr DBL. Subline dbr DBS required only one gene. It is to be noted that the variant sublines were tested a year after the testing of the original spontaneous tumor dbr DM. In another study, Strong (1926a) showed that similar changes in histo-

compatibility genes occurred in still another DBA strain mammary adenocarcinoma such that one subline lost all specifity and grew in all F_2 test mice and mice of all previously resistant strains tested.

The impression is gained that the changes in histocompatibility gene requirements occur rapidly and *en bloc* for example, from a 7 gene-requiring tumor to a 2 gene-requiring tumor, as described above. It should be noted, however, that the changes described, especially in the dbr D tumor, have occurred over the period of a year, representing many cell generations, and could have occurred stepwise and in orderly fashion. In the case of 17495a, a single gene change seems to have occurred as a sudden event (Snell, 1952a).

The mutations which have been reported in transplantable tumors have been in the direction of loss of *H*-gene requirements and thus specificity. The reverse process, increase in specificity, has not been described except in the case of residence of a tumor in an F_1 host (Hauschka, 1953) discussed later. If, however, increase in *H*-gene requirements and specificity signifies reduced growth rate, it is quite probable that the selective advantage would favor the nonmutated cells and such changes would not ordinarily be detected during routine transfers of a tumor.

Additional evidence that the heritable changes observed in various histologic types of tumors, during successive transfers, are genetic in nature is gained from the observations of linkage between a histocompatibility gene and a known marked chromosome carrying dilution, (*d*), Bittner (1933), from sex linkage shown by Strong (1929) and linkage between the *H-2* locus and fused (*Fu*) (Gorer *et al.*, 1948) which will be discussed in detail later.

It seems extremely likely that the mechanism of mutation, described above, resulting in loss of *H*-gene requirements, explains the development of so-called ordinary transplantable tumors such as sarcoma 180 and sarcoma 37. In some cases, however, it is known that tumors, especially sarcomas, are transplantable initially into other inbred strains (Lewis and Lichtenstein, 1938).

Many reports have appeared describing morphologic changes in tumors, especially adenocarcinomas, during the course of transplantation. There is no conclusive evidence that these changes represent true transformations nor that the morphologic changes are in any way related to the physiologic changes described above. The development of sarcomas from a predominant carcinomatous-type tumor, fibrosarcomatous changes in pulmonary adenomas of mice, loss of a keratinizing tendency in squamous cell carcinomas and changes in "virulence" of transplantable leukemias associated with concomitant increase in number of mitochondria per lymphocyte and increase in volume of chromosomes, are examples of morphologic changes that have been described.

Stewart *et al.* (1947) proposed some possible explanations for the type of change observed in transplantable lung adenomas. (1) The lung tumor may have consisted of both carcinomatous and sarcomatous elements; during transplantation the sarcomatous elements may have been selected. (2) A transformation of carcinomatous to sarcomatous cells may have occurred. (3) Nonmalignant stromal cells, derived from the original animal, may have been carried in successive transplants with the malignant cells and become transformed during the process, or (4) sarcomatous transformation of the host cells, in the region of the transplant, may have occurred. Another explanation not given by these authors is that suggested by Nicod (1936). The neoplastic cell may have the capability of producing many different morphologic forms. This would not imply necessarily that different morphologic areas represent cell lineages of different genotypes.

At least one of the alternative explanations given for the observed changes in morphology of tumors is open to experimental investigation. If it is assumed that stromal cells of the host transform to sarcomatous cells during the course of transplantation, it should be possible to determine this by using pulmonary adenomas, for example, originating in pure strain mice, transplanting to F_1 hybrid mice and retransplanting at successive transfers. If sarcomatous transformation of the tumor occurs as a result of transformations of host stromal cells, these tumors should then grow only in the specific F_1 hybrid test mice used and should not ordinarily grow back in the strain of origin. It would also be of interest to determine if any of the numerous morphologic changes in transplantable tumors are related to the genetically detectable physiologic changes which occur.

2. Number of Histocompatibility Genes

Snell (1948) has discussed in detail the manner of estimating the number of *H*-genes concerned in determining compatibility or incompatibility of tumor transplants in inbred strains of mice. It is estimated, from the data available in a large series of crosses, that at least 14 *H*-genes exist in the general mouse population. This estimate is based mainly on tumors in the A and DBA strains. Although in many crosses only a small number of genes is indicated, the result of transfers of tumor in the later transfer passages, the maximum number of *H*-gene differences found in any cross between strains should be used in an estimate. It is obvious, as discussed by Snell, that many factors influence a precise estimate of the number of genes involved. For example: (1) If 2 inbred strains A and B differ by two *H*-genes, there are in all probability many other *H*-genes which they have in common. If the genotype of the A strain is *H1H1*

H2H2 H3H3 h4h4 H5H5 . . . and of the B strain *h1h1 H2H2 H3H3 h4h4 h5h5* . . . , only the genes *H1* and *H5* will be revealed by the cross. In general, any given cross will segregate for only half of the *H*-genes. Thus, if we consider case 5 in Table XIV, it is quite possible that the estimate may be as high as 24 *H*-genes. (2) It has been assumed that *H*-genes for incompatibility are completely recessive, and data obtained in many of the early crosses favored this assumption. However, more recent work by Snell and colleagues shows that in the case of the *H-2* locus, none of the alleles studied thus far behaves as a recessive. (3) The existence of multiple alleles, at least at the *H-2* locus, would increase the chance of segregation for any given locus and thus would increase the number of loci concerned with tumor compatibility. It is most likely that some linkage of these genes must occur since there are 20 pairs of chromosomes in the mouse. Close linkage of 2 *H*-genes would thus appear in the data as one gene. (4) If there exist among tumors arising from different tissues, tissue-specific antigens, which are of course gene determined, similar to the *M* and *N* antigens produced only in erythrocytes this fact would tend to increase any estimate of the number of *H*-genes. Studies to date have not revealed such tissue-specific *H*-genes, but no particular attempt has been made to do so. It is quite likely that they exist.

3. Identification of Specific Histocompatibility Genes

More recent work in the genetics of tumor transplantation has concerned the identification of specific *H*-genes, their distribution in strains of mice and their characterization. The known methods include (1) the use of linked genes as markers and (2) the use of so-called isogenic resistant strains of mice.

The finding by Gorer *et al.* (1948) that one histocompatibility locus, designated *H-2*, was linked with the locus for fused tail *Fu*, provided an excellent method for analysis of such genes. It was shown also that the *H-2* locus had a dual effect: (1) determining a blood group antigen and (2) determining susceptibility or resistance to certain transplantable tumors. Serologic tests done in conjunction with tumor transplantation provided evidence that several alleles were to be found at the locus.

The linkage phenomenon was used to detect new alleles at the *H-2* locus. Crosses of the type:

$$(\mathrm{A} \times Fu) \times \mathrm{B}$$

were used, where A and B are any two inbred strains of mice and *Fu* is a stock carrying the dominant fused, *Fu*, gene. It is also possible to use other stocks of mice in the *Fu* position, carrying the dominant gene *T*, brachyury, which is closely linked with *Fu*. Since the *Fu* stock is hetero-

zygous (*Fu/fu*), F_1 offspring are either normal or fused. Only fused F_1 mice are then crossed to the B strain. Offspring of this double cross are then inoculated with a tumor from the A strain and progressive growth (+) noted. Four classes of offspring are obtained:

$+fu$	$-fu$	$+Fu$	$-Fu$
Susceptible, normal	Resistant, normal	Susceptible, fused	Resistant, fused

If fused tail, *Fu*, shows linkage with resistance, then the A and B strains carry different alleles at the *H-2* locus. If the A and B strains carry the same allele, all mice of the test generation will show progressive growth of the tumor or only part will show progressive growth depending upon segregation of other *H*-genes. No linkage with fused will be found however. If A and B carry different alleles identification and distribution of the alleles can be made by a series of crosses of various inbred strains. Snell and Higgins (1951) and Snell (1951a, 1952b) have demonstrated the existence of at least 6 alleles, $H\text{-}2^{dk}$, $H\text{-}2^{d}$, $H\text{-}2^{k}$, $H\text{-}2^{b}$, $H\text{-}2^{p}$, and $H\text{-}2^{q}$ at the *H-2* locus. Since many other inbred strains of mice remain to be tested, other alleles may yet be revealed. The distribution of these alleles in several inbred strains of mice is shown in Table XVII.

TABLE XVII

Distribution of Different Alleles at *H-2* Locus in Various Inbred Strains of Mice*

$H\text{-}2^{dk}$	$H\text{-}2^{d}$	$H\text{-}2^{k}$	$H\text{-}2^{b}$	$H\text{-}2^{p}$	$H\text{-}2^{q}$
A	BALB/c	C57BR/a	C57BL/10	P	DBA/1
	DBA/2	C57BR/cd	C57BL/6		
	C57BL/10-x†	CBA	C57L		
	C57BL/6-x†	C3H	LP		
		ST	129		
		AKR	Line 11		

* Adapted from data of Snell (1952b).

† Represent mutations from $H\text{-}2^{b}$ to $H\text{-}2^{d}$ in the C57BL strain (Borges, 1952a,b).

Several facts of interest have been revealed by the analysis of the *H-2* locus. Some of these facts may very well explain some of the exceptions to the classical genetic theory of tumor transplantation; others make it necessary to modify the theory insofar as the *H-2* locus is concerned. First, the *H-2* locus is probably the most important locus as far as resistance to tumor growth is concerned. Differences at this locus between the tumor and host prevent progressive growth of the most "virulent" tumors. For example: as shown by Snell and Higgins (1951) A strain tumor, 15091a, a spindle cell carcinoma grew if, and only if, allele $H\text{-}2^{dk}$

were present in the host; DBA strain, lymphocytic leukemia P 1534 and BALB/c strain fibrosarcoma S621 grew only if allele *H-2*^{d} were present; C57BL strain granulocytic leukemia, C1498, grew only if allele *H-2*^{b} were present, and a P strain anaplastic sarcoma grew only if allele *H-2*^{p} were present. In the various crosses used by these authors there was no indication that any histocompatibility locus other than *H-2* was effective in causing resistance to tumor transplants.

Secondly, none of the six alleles found at the *H-2* locus behaves as a recessive, that is, no inbred strain tested so far, carries an allele which is not necessary for the growth of tumors originating in that strain. This is contrary to what was expected based on analysis of published data (Snell, 1948) but is not surprising since the *H-2* allele has been shown by Gorer to determine a series of erythrocytic antigens. This locus thus behaves like the *MN* blood groups in man where both *M* and *N* express themselves when heterozygous. Third, allele *H-2*^{dk}, found only in one inbred strain, so far, behaves as though composed of 2 components, d and k, which are found separately in other strains. All F_1 hybrid mice of the genotype *H-2*^{d}/*H-2*^{k}, produced by crossing a strain carrying the d component (BALB/c or DBA/2) by any strain carrying the k component (the a and cd sublines, of C57Br), AKR, CBA, C3H and ST will grow the spindle-cell mammary carcinoma 15091a, which originated in the A strain. F_1 hybrid mice involving other alleles e.g., BALB/c (*H-2*^{d}) x C57BL (*H-2*^{b}) are negative to the A strain tumor (see Table XVIII).

TABLE XVIII

Data Showing Progressive Growth (+) or no Growth (−) of Spindle Cell Mammary Carcinoma, 15091a, of A Strain origin, in F_1 Hybrid Mice not Involving A (*H-2*^{dk}) Strain*

Case	Cross	Genotypes	Results +	Results −
1	BALB/c x C57BL	*H-2*^{d} x *H-2*^{b}	1	22
2	BALB/c x P	*H-2*^{d} x *H-2*^{p}	1	12
3	C57BL x AKR	*H-2*^{b} x *H-2*^{k}	0	20
4	BALB/c x CBA	*H-2*^{d} x *H-2*^{k}	28	0
5	BALB/c x AKR	*H-2*^{d} x *H-2*^{k}	25	0
6	DBA/2 x AKR	*H-2*^{d} x *H-2*^{k}	14	0

* Adapted from data of Snell (1951a, 1953).

Hauschka and Levan (1952) have also observed the growth of a lymphosarcoma, lymphoma 1, of A strain origin, in F_1 hybrids between DBA/2 (*H-2*^{d}) and C3H (*H-2*^{k}). Fourth, it has been commonly accepted that tumor transplantations to an inbred strain, other than that of origin, fail

to grow or grow temporarily, at least increase in size, and then regress. It would be expected, however, in the case of the *H-2* locus, which is a "strong" locus, that in certain situations tumors arising within a strain bearing a specific *H-2* allele would grow progressively in other strains possessing that specific *H-2* allele. This appears to be true in the case of sarcoma DBA49 (Barrett, 1952) and melanoma S91, both of DBA strain origin, which grew progressively in approximately 90% of mice of the BALB/c strain (Hauschka and Levan, 1952). There are many instances, however, where cross-transplantation between strains with the same *H-2* allele does not occur, indicating the existence of other *H*-loci.

4. Mutations at the H-2 Locus

Haldane (1936) has pointed out, from theoretical considerations, that most members of a mammalian inbred strain will be heterozygous for at least one gene as a result of spontaneous mutation. Some of these genes will appear in the homozygous condition and will become established in the population, giving rise in some cases to sublines within strains. Most of these mutations, apparently have had little relationship to *H*-genes, which influence susceptibility or resistance to tumor transplants. In one case, at least, a mutation has occurred from $H\text{-}2^a$ to $H\text{-}2^d$ or *vice versa*, differentiating subline 1 from subline 2 of the DBA strain (Table XVII).

Borges and Kvedar (1952) have demonstrated experimentally, for the first time, the development of an "isogenic resistant" subline of animals differing from the original strain by a single histocompatibility gene. A new subline, designated C57BL/10-x was developed from the C57BL/10 strain following the discovery that certain mice in a litter failed to grow the granulocytic leukemia, C1498, which originated in this strain and which grew progressively in all C57BL/10 mice through 224 consecutive transfers. Offspring of these resistant mice were found to be totally resistant to C1498 and to other tumors indigenous to the C57BL strain. The resistance pattern to tumors arising in other strains was unchanged except in case 4 Table XIX. A liver carcinoma, C954, in the C57L strain ($H\text{-}2^b/H\text{-}2^b$) grew profusely in the original subline C57BL/10 ($H\text{-}2^b/H\text{-}2^b$) but failed to grow in the mutant subline C57BL/10-x. Subsequent studies by Snell *et al.* (1952) have shown this mutant subline to have changed from $H\text{-}2^b$ to $H\text{-}2^d$.

5. Isogenic Resistant Strains

The identification and characterization of individual *H-2* genes by the method of linkage studies, discussed above, is limited to cases where linkage can be demonstrated. Another method for the study of such genes is through the production of "isogenic resistant" (IR) strains of mice.

TABLE XIX

Progressive Growth (+), or no Growth (−) of Various Transplantable Tumors in C57BL/10 and in Mutant Strain C57BL/10-x Mice*

Case	Tumor Designation	Tumor Type	Strain of Origin	Number of Mice				Per Cent progressive growth	
				C57BL/10		C57BL/10-x			
				+	−	+	−	C57BL/10	C57BL/10-x
1	C-1498	Granulocytic leukemia	C57BL/6†	259	0	0	612	100	0
2	BL	Breast carcinoma	C57BL/6	31	0	0	63	100	0
3	S653	Rhabdomyosarcoma	C57BL/10	41	0	0	67	100	0
4	C-954	Liver carcinoma	C57L‡	63	36	0	112	63.6	0
5	dbr B	Breast carcinoma	DBA	0	50	0	89	0	0
6	15091A	Breast carcinoma	A	0	16	0	29	0	0
7	P1534	Lymphocytic leukemia	DBA/2	0	72	3	100	0	2.9

* Adapted from data of Borges and Kvedar (1952).

† Sublines 6 and 10 of the C57BL strain have the same H-2 allele, $H\text{-}2^b$. Tumors rising within either strain are transplantable to the other.

‡ The C57L strain has the $H\text{-}2^b$ allele.

More important is the development of such strains for studies in tumor immunity.

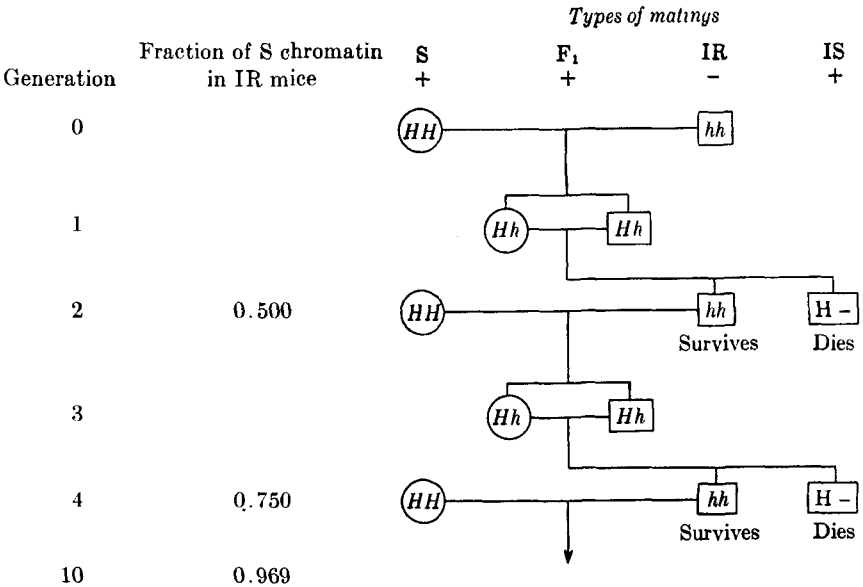
It has been suggested by many investigators that regressing transplanted tumors in resistant hosts may be explained as an immunity phenomenon. The tumor contains an antigen (antigens) which evokes the formation of an antibody (antibodies) causing destruction of the grafted tumor. Since it has been shown that tumor susceptibility and resistance may be traced to specific genes, it is highly likely that the antigens involved are more or less direct products of these genes. The estimate given for the number of *H*-loci is not less than 14 and probably much greater. Thus, if one assumes that the number of antigens related to tumor regression is of the same order as the number of gene loci, and this seems to be the case with blood groups in man, it may be seen that a complex antigenic situation results even with the use of inbred strains of mice. Two inbred strains are seen to differ by as many as 8–11 histocompatibility loci. An analysis of the antigens and antibodies would be nearly impossible. A favorable situation then for analysis is established by the production of isogenic resistant (IR) sublines of mice. When a tumor indigenous to an inbred strain *HH*, regresses in a mouse of the isogenic resistant subline, *hh*, it is a reasonable assumption that regression is due to the action of a single antigen produced by the gene *H*. It is also reasonable to assume that preparations made from the tumor contain only one protein foreign to the IR subline since this IR subline differs from the inbred strain by a single histocompatibility gene. Serologic reactions thus become specific tests for a single substance and a controlled situation is thereby created for the study of an *H*-gene and its products. Thus, the isolation, physically and chemically, of cellular constituents involved in tumor immunity phenomena may be accomplished, their distribution studied and identification of various antigens established.

The detection of somatic mutations in transplantable tumors resulting in loss of specificity is simplified by the use of IR strains of mice. A tumor arising, for example, in C3H strain mice which will grow progressively only in the presence of gene *Hx* would fail to grow completely, or nearly so, in an isogenic resistant C3H subline genetically *hx hx*. Should a mutation occur in the tumor so that gene *Hx* is no longer required, progressive growth will then be obtained in 100% of the IR line. In such an experimental setup a study of specific agents inducing mutations in tumors, and in normal tissues as well, is possible.

Some 70 isogenic resistant strains have now been developed and identification and characterization of specific *H*-genes is in progress. The reader is referred to Snell (1948) for experimental details, and to Fig. 4, showing the method used in establishing IR strains of mice.

Identification of 2 additional *H*-loci, designated *H-1* and *H-3* have been made to date using IR strains of mice (Snell, 1953a). Appropriate tests have shown the existence of an allele of the *H-1* locus, *H-1*^{a}, in the C3H strain. This allele is found in the first linkage group of the mouse and is

Method used for introducing a histocompatibility gene (*h*) into an inbred strain (S), genetically (*HH*) to produce a resistant subline (IR), genetically (*hh*) but otherwise identical with the S strain*



* Adapted from material of Snell (1948).

FIG. 4. This method consists of the production of F_2 hybrid individuals from the cross of a susceptible strain S, for example the DBA strain, which will grow a DBA tumor, by a resistant strain, for example, the C57BL strain which resists the tumor. Mice of the F_2, F_4, F_6 etc. generations are inoculated with the DBA tumor and the survivors (*hh*) are again mated to the S strain. It may be seen that after 10 generations the chromation of the isogenic resistant subline (IR) is 96.9% from the S strain. After 10 generations the *IR* mice are then mated *interse*.

linked with albinism (*c*), giving a cross-over per cent of 26.6 ± 3.7 between *H-1* and *c*. The DBA/2 strain carries the same *H-1*^{a} allele, but strains A, BALB/*c*, C57BL, C57BR, and C57L are not *H-1*^{a} (Snell and Kelton, 1953b).

6. Antigenic Basis of Tumor Immunity

Numerous studies have been reported in an attempt to disclose the presence of circulating and more specifically "humoral" antibodies to

tumor or tissue transplants. For a complete review of the subject the work of Woglom (1929) and Spencer (1942) and more recently Hauschka (1952) and Snell (1952a) should be consulted. Many of these reports (Kidd, 1946; Burmester, 1947; Lumsden, 1937) have dealt with specific tumor antigens. Since, in most studies, heterogeneous strains of animals were used, and genetic differences between host and tumor existed, it is quite apparent that specific antigenic entities could not be distinguished. The existence of Wassermann and Forssman antigens, tissue—and organ—specific antigens, *H*-gene antigen, blood group substances, the presence of viruses and bacteria as contaminants, etc., may all lead to nonspecific reactions.

The work of Gorer (1937, 1938, 1942) is considered pertinent because of the use of stringent genetic controls and combined transplantation, hemagglutination and differential absorption techniques. Mice of the C57BL strain were hyperimmunized by inoculation of leukemic cells of an A strain lymphocytic leukemia. Immunization resulted in the formation of hemagglutinins. When the hyperimmune serum was mixed with leukemic cells and inoculated into A strain mice, growth of the leukemic cells was retarded, or in some cases, completely inhibited as determined by organ infiltrations or survival time. C57BL serum obtained from uninoculated mice was ineffective. The protective antibody could be completely removed by absorption with leukemic cells of the A strain and removed partially by erythrocytes of the A strain only. Thus, it was concluded that erythrocytes and tumor cells share a common antigen, which was called antigen II. This antigen could also be demonstrated in rabbits by the production of antisera following injections of A strain erythrocytes. The identity of the antigen reacting with antisera produced by either method was established by testing antisera against erythrocytes of F_2 hybrid mice (A & C57BL strains). The manner of inheritance of antigen II and its relation to growth of the tumor was determined in F_2 and backcross generations of those strains. The presence of antigen was determined by agglutination and then mice were inoculated with tumor. The results of similar tests using a mammary carcinoma of the A strain are shown in Table XX.

It may be seen that close agreement is obtained on the assumption that two *H*-genes were segregating in the F_2 and backcross generation, one of which determined antigen II. Serologic results support this conclusion. Mice lacking antigen II failed to grow the tumor; some of the mice with antigen II also failed to grow the tumor probably the result of not having a second segregating *H*-gene. The gene determining antigen II has been called *H-2*.

As described above the *H-2* locus has been studied extensively in genetic tests because of its close linkage with fused tail (*Fu*). This locus

plays a dual role in determining erythrocytic antigens and susceptibility or resistance to transplantable tumors. A relationship remains to be established between the agglutinins and antibodies which cause inhibition of the tumor transplants. Ordinary agglutinins appear to have no protective functions. As shown by Gorer (1942) the inhibitory function was not removed by absorption of agglutinins. Thus, it was concluded that non-agglutinating antibodies also probably exist which could be absorbed by tumor cells, It has been suggested by Gorer (1948) that ordinary agglutinins without protective function may mature to a functional antibody.

TABLE XX

Results of Agglutination and Tumor Inoculation Tests in F_2 and Backcross Mice Produced by Crossing A and C57BL Strains*

	II	II	Not II	Not II
	+	−	+	−
F_2 observed	35	13	0	17
F_2 expected	36.6	12.2	0	16.2
Backcross observed	17	17	0	44
Backcross expected	19.5	19.5	0	39

Note. Mammary carcinoma of the A strain used. + = progressive growth, − = no growth of tumor. The results are compared with expectations if 2 *H*-genes determine susceptibility to the growth of tumor.

* Data of Gorer, 1937.

Nevertheless, both types of antibody seem directed against the same antigen.

It seems justified to conclude then that probably all *H*-genes are related to antigens although serological identification remains to be accomplished.

7. Induced Immunity and Enhancement

It has been shown, through the use of various host-tumor combinations, that neoplastic growth may be completely prevented, partially inhibited or, in fact, greatly enhanced. The tissue used to produce these responses need not be neoplastic nor homologous with the tumor used. Embryonic tissue, blood and nonliving material will cause inhibition of growth of transplanted tumors in certain situations. It is known, too, that such inhibition of growth is not obtained when tumor and host are from the same inbred strain or more specifically from the same subline of the same inbred strain. The several cases proving exceptions to this rule have not been controlled rigidly from a genetic standpoint and either strain or

tumor have mutated during the long period of serial transfer. Snell *et al.* (1946) and Hauschka (1952) have discussed these exceptions in detail.

These results would suggest that the mechanisms encountered in immunity to neoplasm are not specifically antineoplastic, but probably are the result of isoantigenic differences as postulated by Gorer (1942). Thus, too much weight cannot be given to studies of tumors arising in heterogeneous animals and serially transferred in these and other hosts. It would be expected that antibodies produced in any such experimental situation would be antibodies not directed against neoplastic cells as such but against all cells which are genetically unlike the neoplasm.

Recent studies by Snell (1952b) on the "enhancing" effect are of interest. Nonliving tissue or tissue products are known to produce inhibition in transplantable tumors (Snell *et al.*, 1946). . . . In certain host-tumor combinations it was found that injections of lyophilized tumor tissue or lyophilized normal tissue produced an enhancing effect such that tumors which originally failed completely to grow, grew progressively in mice given prior injections of the lyophilized tissues. Two tumors, L946, a fibrosarcoma, and E0771, a mammary carcinoma, inhibited growth of the challenge tumor in certain inbred strains but enhanced growth in other genotypes. Enhancement was also obtained by the use of cell fractions obtained by differential centrifugation (Snell, 1951b) or by tissue antisera (Kaliss and Molomut, 1952), although in the latter case the enhancing effect was slight. It was reported that the difference between resistance and enhancement, at least in one host-tumor situation was a dosage phenomenon; small doses of antigen giving resistance, large doses, enhancement. This is an interesting observation and should be repeated in view of the inability of Barrett (1952) to obtain a dosage effect using the erythrocytic antigen of sarcoma DBA 49 grown in BALB/c mice. Not only is the enhancing effect described by Snell found to be species-specific, but apparently bears a close relationship to histocompatibility genes described earlier. If the spindle cell carcinoma, 15091a, of A strain origin ($H\text{-}2^{dk}$-$H\text{-}2^{dk}$), is inoculated into either C57Br strain ($H\text{-}2^{k}H\text{-}2^{k}$) or C57BL strain mice ($H\text{-}2^{b}H\text{-}2^{b}$), both resistant strains, enhancement of growth was found to be greatest with prior injections of lyophilized tissue having the same *H-2* component (*dk*) as the tumor tissue. Minimum effects were found with C57Br tissue ($H\text{-}2^{k}H\text{-}2^{k}$) in C57Br hosts and with P strain tissue ($H\text{-}2^{p}H\text{-}2^{p}$) in either of the two hosts. (See Table XXI.) These data, according to Snell, are compatible with the assumption that enhancement depends upon pretreatment with the histocompatibility genes (or their products) which are present in the tumor being tested but absent from and hence foreign to the host. The results may be coincidental, however, and

should be corroborated. The use of isogenic resistant strains should give an unequivocal answer.

TABLE XXI

Percentage Progressive Growth of Tumor 15091a of A Strain Origin Following Prior Injections into Resistant Hosts of Lyophilized Normal Tissues from Various Inbred Strains*

	Donor Strain†						
Host Strain	A (dk)‡	BALB/c DBA/2 (d)	CBA ST (k)	C57BL (b)	P (p)	C57BR (k)	No Tissue
C57BL (b)‡	50	6	40	—	0	—	0
C57Br (k)	44	45	29	31	11	12	4

* Adapted from data of Snell (1952b).
† Normal tissues used were kidney and spleen.
‡ Components of *H-2* loci.

8. Variability of Tumor Cell Populations

The variability of inbred strains of mice in regard to identifiable *H*-genes and the identification of mutant variants in transplantable tumors have been discussed. Many facts, some not generally recognized, indicate the complex mosaic of any given neoplastic cell population and the result of selective pressures brought to bear either as a natural consequence or by experimental interference. The development of so-called (1) autonomy in tumors, (2) the morphologic transitions from one predominant cell type to another, (3) changes of antigenic specificity, (4) changes from types showing some degree of differentiation to complete anaplasticity, and (5) shifts in chromosome ploidy are examples of the modification of the behavior of tumor cell populations.

By use of the "fluctuation test" it has been shown by Law (1952c) that the variant resistant and dependent leukemic cell populations, developed by the use of antifolic and antipurine compounds, represent stable, irreversible, and heritable changes which occur as mutants in the absence of these antileukemic agents, but are selected out by the agents. Such a test is not limited to resistance studies but is readily adaptable to a study of changes in immunogenetic, morphologic, and functional characteristics of transplantable tumors.

Abrupt changes in the specificity of tumors, as determined by their transplantability in resistant backcross hybrids, have been observed by transferring for one passage through an F_1 hybrid host between the sus-

ceptible strain and a resistant strain. In the case of an adenocarcinoma of the breast (C3HBA) in C3H strain mice, Barrett and Deringer (1950) have observed a 3-fold increase in backcross "takes" after residence in an F_1 hybrid host. This altered transplantability was found to be permanently fixed (Barrett and Deringer, 1952). Significant changes in the transplantability of two other tumors, the TA3 mammary adenocarcinoma, and the DBA lymphoma, were observed by Hauschka (1953) following this same method of passage through an F_1 hybrid. In this study, an increase as well as a decrease in specificity was observed. It is of interest to note, also, the difference in behavior of the DBA lymphoma when observed either as a solid tumor, grown subcutaneously or as an ascites tumor. The observed differences may indicate an influence of transplantation site, stroma, or physical state on the antigenic properties of the tumor. Some complex immunologic selection would appear to be effective in these observed transformations. Good agreement is obtained in some instances between the modified percentages of "take" and Mendelian ratios, and it is possible that simple shifts occur in *H*-gene requirements, for example, from a 3-gene requiring tumor to a 2-gene requiring tumor or in the opposite direction in case of increase in specificity. Irwin (1947) has shown in certain species-hybrids, in crosses between dove and pigeon, that antigenic substances different from and in addition to the parental antigens are found. In an F_1 hybrid, recessive *h*-genes or partially dominant *H*-genes, not found in the parental strain, may be effective as selective agents in elaborating antibodies affecting the tumor graft by favoring variants with either more or fewer *H*-genes than the common cell type. The interaction of genes, which are neutral in the parental strain in which the tumor arose but active in the F_1 heterozygote could clearly modify immunologic responses. Gorer (1948) suggests that decreased host specificity, at least in regard to consecutive transfers of transplantable tumors, is the result of certain antigens crowding out others and that this masking explains the functional elimination of the weaker *H*-gene effects. Thus, the genotype of a transplantable tumor need not necessarily change. The problem to be solved in this modification of tumor transplantability following residence in an F_1 hybrid is: Is this a genuine adaptive response or is there a selective mechanism in the environment of the heterozygote shifting the equilibrium toward decreased or increased specificity? The latter explanation seems the more likely and, if so, identification of the type of change and the selective mechanisms involved should be made possible by the "fluctuation test" or by the micro-isolation procedures of Hauschka (1953).

Similar changes in transplantability of tumors, have been observed following foster-nursing (Cloudman, 1941a; Law, 1942; Barrett and Mor-

gan, 1949) or by injections of tissue extracts and filtrates from susceptible to resistant mice (Law, 1944).

9. Chromosome Ploidy and Transplantability of Tumors

Generally, neoplasms which have been analyzed for chromosome numbers are found to be chromosomal mosaics with a wide range of chromosome numbers extending far below the $2n$ (diploid) to far above the $4n$ (tetraploid) types (Hauschka and Levan, 1953, 1951; Koller, 1947). Distinct diploid or tetraploid modes were characteristic of specific tumors and those cells with the most balanced genome either $2n$ or $4n$, appeared to serve as "stem cells" in the propagation of tumors through consecutive transfers. Nevertheless, many viable cells with disturbed chromosome numbers are found at any time within a tumor cell population and provide a source of genetic variation (Hauschka, 1953).

Various $2n$ (diploid) and $4n$ (tetraploid) tumors of different histologic type and virulence and from many different inbred strains of mice were examined for transplantability in many host strains by Hauschka and Levan (1953), and Hauschka (1953). A consistent relationship was found between chromosome constitution of the tumor cells and transplantability. All tumors known to be diploid were strain-specific; the tetraploid tumors showed varying degrees of genetic indifference in regard to their host requirements, growing for the most part in many different unrelated strains. The common transplantable tumors S180, S37, etc., were found to be tetraploid types.

The wider range of transplantability of the tetraploid tumors was interpreted as a result of a disturbed genic balance. In a polyploid tumor cell, more than 2 allelic H-genes may function in dosages other than those to which they are restricted in a diploid cell, so that antigens may no longer elicit adequate protective antibodies. Thus, loss of specificity is viewed as selection of the least antigenic or less compatible types from among the numerous chromosomal variants in a tumor. Alternatively, an explanation for the loss of specificity may be a mutation from $H \rightarrow h$. The mutation rate may be expected to double in a tetraploid tumor. It would be of interest to follow the characteristics of a polyploid tumor from the time of origin through consecutive serial passages in an attempt to answer some of the pertinent questions.

A simple change from $2n$ to $4n$ is probably not the essential factor in loss of specificity of tumors. If this were so, tetraploid cells in a predominantly diploid tumor cell population, should survive and give rise to a different, that is a $4n$ tumor, when transplanted into a foreign host. This has not been accomplished experimentally. Apparently duplication, followed by "immunologic selection" is necessary.

Additional evidence for the role of ploidy in overcoming the barrier of tumor host incompatibility is being obtained through the establishment of single-cell clonal sublines of ascites tumors (Hauscha, 1953).

REFERENCES

Andervont, H. B. 1937a. *Public Health Repts. U.S.* **52,** 304–315.
Andervont, H. B. 1937b. *Public Health Repts. U.S.* **52,** 1584–1589.
Andervont, H. B. 1937c. *Public Health Repts. U.S.* **52,** 212–221.
Andervont, H. B. 1937d. *Public Health Repts. U.S.* **52,** 304–315.
Andervont, H. B. 1938a. *Public Health Repts. U.S.* **53,** 232–237.
Andervont, H. B. 1938b. *Public Health Repts. U.S.* **53,** 1647–1665.
Andervont, H. B. 1939. *Public Health Repts. U.S.* **54,** 1158–1169.
Andervont, H. B. 1940a. *J. Natl. Cancer Inst.* **1,** 147–153.
Andervont, H. B. 1940b. *J. Natl. Cancer Inst.* **1,** 135–145.
Andervont, H. B. 1941. *J. Natl. Cancer Inst.* **1,** 737–744.
Andervont, H. B. 1945a. *J. Natl. Cancer Inst.* **5,** 391–396.
Andervont, H. B. 1945b. Mammary Tumors in Mice. *Publ. Am. Assoc. Advance. Sci.,* Washington, D.C., pp. 123–139.
Andervont, H. B. 1945c. *J. Natl. Cancer Inst.* **5,** 383–390.
Andervont, H. B. 1949. *J. Natl. Cancer Inst.* **10,** 201–214.
Andervont, H. B. 1950. *J. Natl. Cancer Inst.* **11,** 73–81.
Andervont, H. B. 1952. *Ann. N. Y. Acad. Sci.* **54,** 1004–1011.
Andervont, H. B., and Bryan, W. R. 1944. *J. Natl. Cancer Inst.* **5,** 143–149.
Andervont, H. B., and Dunn, T. B. 1948. *J. Natl. Cancer Inst.* **8,** 227–233.
Andervont, H. B., and Dunn, T. B. 1950a. *J. Natl. Cancer Inst.* **10,** 895–926.
Andervont, H. B., and Dunn, T. B. 1950b. *J. Natl. Cancer Inst.* **10,** 1157–1189.
Andervont, H. B., and Dunn, T. B. 1952. *J. Natl. Cancer Inst.* **13,** 455–485.
Andervont, H. B., and Shimkin, M. B. 1940. *J. Natl. Cancer Inst.* **1,** 225–239.
Andervont, H. B., and Stewart, H. L. 1937. *Science* **86,** 566–567.
Armstrong, E. C., and Bonser, G. M. 1947. *J. Pathol. Bacteriol.* **59,** 19–27.
Armstrong, E. C. 1948. *Brit. J. Cancer* **2,** 59–69.
Auerbach, C. 1949. *Biol. Revs. Cambridge Phil. Soc.* **24,** 355–391.
Barnum, C. P., Ball, Z. B., Bittner, J. J., and Visscher, M. B. 1944. *Science* **100,** 575–576.
Barnum, C. P., Ball, Z. B., and Bittner, J. J. 1946. *Cancer Research* **6,** 499–503.
Barnum, C. P., Ball, Z. B., and Bittner, J. J. 1947. *Cancer Research* **7,** 522–528.
Barnum, C. P., Huseby, R. A., and Bittner, J. J. 1948. *Acta Union contre le cancer* **6,** 185–186.
Barrett, M. K. 1952. *Cancer Research* **12,** 535–542.
Barrett, M. K., and Deringer, M. K. 1950. *J. Natl Cancer Inst.* **11,** 51–59.
Barrett, M. K., and Deringer, M. K. 1952. *J. Natl. Cancer Inst.* **12,** 1011–1017.
Barrett, M. K., and Morgan, W. C 1949. *J. Natl. Cancer Inst.* **10,** 81–88.
Bashford, E. F., Murray, J. A., Haaland, M., and Bowen, W. H. 1908. Third Sci. Rept. Cancer Res. Fund, London, pp. 262–265.
Bennison, B. E. 1948. *Acta Union contre le cancer* **6,** 188.
Bierman, H. R., Byron, R. L., Kelly, K. H., and Cordes, F. 1951. *Cancer Research* **11,** 236–242.
Bittner, J. J. 1932. *Am. J. Cancer* **17,** 1144–1152.
Bittner, J. J. 1933. *Am. J. Cancer* **17,** 699–708.

Bittner, J. J. 1935. *J. Genetics* **31,** 471–487.
Bittner, J. J. 1936. *Science* **84,** 162–163.
Bittner, J. J. 1937a. *Heredity* **27,** 391–396.
Bittner, J. J. 1937b. *Am. J. Cancer* **30,** 530–536.
Bittner, J. J. 1937c. *Am. J. Clin. Pathol.* **7,** 430–435.
Bittner, J. J. 1938. *Public Health Repts.* **53,** 2197–2202.
Bittner, J. J. 1939a. *Public Health Repts.* **54,** 1113–1118.
Bittner, J. J. 1939b. *Am. J. Cancer* **35,** 90–97.
Bittner, J. J. 1940. *J. Natl. Cancer Inst.* **1,** 155–163.
Bittner, J. J. 1941. *Cancer Research* **1,** 113–114.
Bittner, J. J. 1942a. *Science* **95,** 462–464.
Bittner, J. J. 1942b. *Cancer Research* **2,** 755–777.
Bittner, J. J. 1944. *Cancer Research* **4,** 159–167.
Bittner, J. J. 1945. Research Conference on Cancer. *Publ. Am. Assoc. Advance. Sci.* pp. 63–96.
Bittner, J. J. 1948. *Proc. Soc. Exptl. Biol. Med.* **67,** 219–221.
Bittner, J. J. 1950. *Cancer Research* **10,** 204.
Bittner, J. J. 1952. *Texas Repts. Biol. Med.* **10,** 160–166.
Bittner, J. J., and Huseby, R. A. 1946. *Cancer Research* **6,** 235–239.
Bittner, J. J., Huseby, R. A., Visscher, M. B., Ball, Z. B., and Smith, F. 1944. *Science* **99,** 83–85.
Bittner, J. J., and Little, C. C. 1937. *J. Heredity* **28,** 117–121.
Blackmore, F. 1934. *4th Rept. Univ. Cambridge Inst. Animal Pathol.* p. 39.
Borges, P. R. F., and Kvedar, B. J. 1952a. *Cancer Research* **12,** 19–24.
Borges, P. R. F. 1952b. Personal communication.
Burdette, W. J. 1943. *Cancer Research* **3,** 318–320.
Burmester, B. R., and Belding, T. C. 1947. *Am. J. Vet. Research* **8,** 128–133.
Burns, E. L., Moskop, M., Suntzeff, V., and Loeb, L. 1936. *Am. J. Cancer* **26,** 56–68.
Chao, P. K. 1952. *Proc. Natl. Acad. Sci.* (In press.) See reference in Sonneborn, 1951.
Cloudman, A. M. 1932. *Am. J. Cancer* **16,** 568–630.
Cloudman, A. M. 1941a. *Science* **93,** 380–381.
Cloudman, A. M. 1941b. Biology of the Laboratory Mouse. The Blakiston Co., Philadelphia, Pa.
Cole, R. K., and Furth, J. 1941. *Cancer Research* **1,** 957–965.
de Bruyn, W. M. 1949. *Cancer Research* **9,** 395–397.
de Bruyn, W. M., Korteweg, R., and van Waveren, E. K. 1949. *Cancer Research* **9,** 282–293.
Demerec, M. 1947. *Nature* **159,** 604.
Demerec, M. 1948. *Genetics* **33,** 337–342.
Deringer, M. K., Heston, W. E., and Andervont, H. B. 1945. *J. Natl. Cancer Inst.* **5,** 403–405.
Dmochowski, L. 1944. *Brit. J. Exptl. Pathol.* **25,** 138–143.
Dmochowski, L., and Orr, J.W. 1949. *Brit. J. Cancer* **3,** 376–384.
Dmochowski, L., and Passey, R. D. 1952. *Ann. N.Y. Acad. Sci.* **54,** 1035–1066.
Dunn, T. B. 1945. Mammary Tumors in Mice. *Publ. Am. Assoc. Advance. Sci.* **22,** 13–38.
Dunn, T. B. 1951. *J. Am. Med. Women's Assoc.* **6,** 411–418.
Dunn, T. B. 1953. To be published in *J. Natl. Cancer Inst.*
East, E. M. 1915. *Genetics* **1,** 164–176.
Eberth, C. J. 1878. *Arch. Pathol. Anat.* **72,** 108.

Edwards, J. E. 1941. *J. Natl. Cancer Inst.* **2**, 197–199.
Edwards, J. E., and Dalton, A. J. 1942a. *J. Natl. Cancer Inst.* **3**, 19–41.
Edwards, J. E , Heston, W. E., and Dalton, A. J. 1942b. *J. Natl. Cancer Inst.* **3**, 297–301.
Engelbreth-Holm, J. 1940. *Cancer Research* **1**, 109–112.
Engelbreth-Holm, J 1942. Leukaemia in Animals. Oliver & Boyd, London.
Engelbreth-Holm, J., and Frederiksen, O. 1938. *Acta. Pathol. Scand.* **37**, 145.
Fekete, E. 1953. Personal communication.
Fekete, E., and Little, C. C. 1942. *Cancer Research* **2**, 525–530.
Foster, C. G., and Miller, F. R. 1950. *Proc. Soc. Exptl. Biol. Med.* **75**, 633–636.
Foulds, L. 1949. *Brit. J. Cancer* **3**, 230–239.
Furth, J. 1946a. *J. Geriatrics* **1**, 46–54.
Furth, J. 1946b. *Physiol. Revs.* **26**, 47–76.
Furth, J. 1951. *Blood* **6**, 964–975.
Furth, J., and Barnes, W. A. 1941. *Cancer Research* **1**, 17–22.
Furth, J., and Boon, M. C. 1945. Research Conference on Cancer. *Publ. Am. Assoc. Advance. Sci.* pp. 129–138.
Furth, J., Boon, M. C., and Kaliss, N. 1944. *Cancer Research* **4**, 1–10.
Furth, J., Cole, R. K., and Boon, M. C. 1942. *Cancer Research* **2**, 280–283.
Furth, J., Ferris, H. W., and Reznikoff, P. 1935 *J. Am. Med. Assoc.* **105**, 1824.
Furth, J., and Kahn, M. C. 1937. *Am. J. Cancer* **31**, 276–282.
Gardner, W. U. 1939. *Arch. Path.* **27**, 138–170.
Gardner, W. U., Dougherty, T. F., and Williams, W. L. 1944. *Cancer Research* **4**, 73–87.
Gordon, M. H. 1936. See annotation in *Lancet* **260**, 158 (1951).
Gorer, P. A. 1937. *J. Pathol. Bacteriol.* **44**, 691–697.
Gorer, P. A. 1938. *J. Pathol. Bacteriol.* **47**, 231–252.
Gorer, P. A. 1940. *J. Pathol. Bacteriol.* **50**, 17–24.
Gorer, P. A. 1942. *J. Pathol. Bacteriol.* **54**, 51–65.
Gorer, P. A. 1948. *Brit. J. Cancer* **2**, 103–107.
Gorer, P. A., and Law, L. W. 1949. *Brit. J. Cancer* **3**, 90–92.
Gorer, P. A., Lyman, S., and Snell, G. D. 1948. *Proc. Royal Soc. (London)* **B135**, 499–505.
Grady, H. C., and Stewart, H. L. 1940. *Am. J. Pathol.* **16**, 417–432.
Graff, S., Heidelberger, M., and Haagensen, C. 1952. *Ann. N.Y. Acad. Sci.* **54**, 1012–1017.
Green, R. G., and Bittner, J. J. 1946. *Cancer Research* **6**, 499–503.
Green, R. G., Moosey, M. M., and Bittner, J. J. 1946. *Proc. Soc. Exptl. Biol. Med.* **61**, 115–118.
Greig, J. R. 1940. *Trans. Highland and Agr. Soc. Scot.* **52**, 71–75.
Gross, L. 1950. *Cancer* **3**, 1073–1087.
Gross, L. 1951a. *Proc. Soc. Exptl. Biol. Med.* **76**, 27–32.
Gross, L. 1951b. *Proc. Soc. Exptl. Biol. Med.* **78**, 342–348.
Gross, L. 1952. *Ann. N.Y. Acad. Sci.* **54**, 1184–1196.
Gross, L. 1953. *Cancer* **6**, 153–158 (1953).
Grüneberg, H. 1952a. Genetics of the Mouse. Martius Nijhoff, The Hague
Grüneberg, H. 1952b. *J. Genetics* **51**, 95–114.
Haaland, M. 1911. 4th Rept. Cancer Research Fund. p. 1.
Haldane, J. B. S. 1936. *J. Genetics* **32**, 375–391.
Hauschka, T. S. 1952. *Cancer Research* **12**, 615–633.
Hauschka, T. S. 1953. *J. Natl. Cancer Inst.* **13** (In press.)

Hauschka, T. S., and Levan, A. 1951. *Anat. Record* **111,** 467.
Hauschka, T. S., and Levan, A. 1953. *Exptl. Cell. Research* **4,** 457.
Heston, W. E. 1940. *J. Natl. Cancer Inst.* **1,** 105–111.
Heston, W. E. 1941. *J. Natl. Cancer Inst.* **2,** 127–132.
Heston, W. E. 1942a. *J. Natl. Cancer Inst.* **3,** 69–78.
Heston, W. E. 1942b. *J. Natl. Cancer Inst.* **3,** 79–82.
Heston, W. E. 1942c. *J. Natl. Cancer Inst.* **3,** 303–308.
Heston, W. E. 1944. *J. Natl. Cancer Inst.* **5,** 161–171.
Heston, W. E. 1945. Mammary Tumors in Mice. *Publ. Am. Assoc. Advance. Sci.* **22,** 55–82.
Heston, W. E. 1948. *Advances in Genet.* **2,** 99–126.
Heston, W. E., 1949a. Roscoe B. Jackson Memorial Laboratory. Twentieth Commemoration 9–31. Publ. Bar Harbor Times, Bar Harbor, Me.
Heston, W. E. 1949b. *J. Natl. Cancer Inst.* **10,** 125–130.
Heston, W. E. 1950. *J. Natl. Cancer Inst.* **11,** 415–423.
Heston, W. E. 1951. *Growth* **10,** 23–46.
Heston, W. E., and Andervont, H. B. 1944. *J. Natl. Cancer Inst.* **4,** 403–407.
Heston, W. E., and Deringer, M. K. 1947. *J. Natl. Cancer Inst.* **7,** 463–465.
Heston, W. E., and Deringer, M. K. 1948. *Acta Union contre le cancer* **6,** 262–263.
Heston, W. E., and Deringer, M. K. 1949. *J. Natl. Cancer Inst.* **10,** 119–124.
Heston, W. E., Deringer, M. K., and Andervont, H. B. 1945. *J. Natl. Cancer Inst.* **5,** 289–307.
Heston, W. E., Deringer, M. K., Dunn, T. B., and Levillain, W. D. 1950. *J. Natl. Cancer Inst.* **10,** 1139–1155.
Heston, W. E., Deringer, M. K., Hughes, I. R., and Cornfield, J. 1952. *J. Nat. Cancer Inst.* **12,** 1141–1157.
Heston, W. E., and Dunn, T. B. 1951. *J. Natl. Cancer Inst.* **11,** 1057–1071.
Huseby, R. A., and Bittner, J. J. 1950. A Symposium on Steroid Hormones, pp. 363–373. University of Wisconsin Press, Madison, Wis.
Huseby, R. A., Smith, F. W., and Bittner, J. J. 1946. *Cancer Research* **6,** 494.
Imagawa, D., Green, R. G., and Halvorson, H. O. 1948. *Proc. Soc. Exptl. Biol. Med.* **68,** 162–165.
Imagawa, D., Syverton, J. T., and Bittner, J. J. 1951. *Cancer Research* **11,** 259–264.
Irwin, M. R. 1947. Immunogenetics. *Advances in Genet.* **1,** 133–159.
Jones, E. E. 1940. *Am. J. Cancer* **39,** 94–99.
Kaliss, N., and Molomut, N. 1952. *Cancer Research* **12,** 110–112.
Kaplan, H. S. 1949. *J. Natl. Cancer Inst.* **10,** 267–270.
Kaplan, H. S. 1950. *J. Natl. Cancer Inst.* **11,** 83–90.
Kaplan, H. S. 1951. *Cancer Research* **11,** 261.
Kidd, J. G. 1946. *Cold Spring Harbor Symposia Quant. Biol.* **11,** 94–110.
Kirby, A. H. M. 1945. *Cancer Research* **5,** 673–682.
Kirschbaum, A., 1949. *Cancer Research* **9,** 93–95.
Kirschbaum, A., and Strong, L. C. 1942. *Proc. Soc. Exptl. Biol. Med.* **51,** 404–406.
Kirschbaum, A., Williams, W. L., and Bittner, J. J., 1946. *Cancer Research* **6,** 354–362.
Klein, G. 1952. *Cancer Research* **12,** 589–590.
Koller, P. C. 1947. *Brit. J. Cancer* **1,** 38–47.
Korteweg, R. 1929. *Z. Krebsforsch.* **29,** 455.
Korteweg, R. 1934. *Ned. Tijdschr. Geneesk.* **78,** 240–245.
Lacassagne, A. 1934. *Compt. rend. soc. biol.* **115,** 937–939.
Law, L. W. 1941. *Proc. Soc. Exptl. Biol. Med.* **48,** 486–487.

Law, L. W. 1942. *Cancer Research* **2,** 108–115.
Law, L. W. 1944. *Cancer Research* **4,** 257–260.
Law, L. W. 1951. *Cancer Research* **11,** 795–800.
Law, L. W. 1952a. *J. Natl. Cancer Inst.* **12,** 1119–1126.
Law, L. W. 1952b. *J. Natl. Cancer Inst.* **12,** 789–805.
Law, L. W. 1952c. *Nature* **169,** 628–629.
Law, L. W. 1953. *Ann. N.Y. Acad. Sci.* In press.
Law, L. W., and Boyle, P. J. 1953. Unpublished observations.
Law, L. W., and Dunn, T. B. 1951. *J. Natl. Cancer Inst.* **11,** 1037–1055.
Law, L. W., and Malmgren, R. 1951. *J. Natl. Cancer Inst.* **6,** 1259–1268.
Law, L. W., and Miller, J. H. 1950a. *J. Natl. Cancer Inst.* **11,** 425–437.
Law, L. W., and Miller, J. H. 1950b. *J. Natl. Cancer Inst.* **11,** 253–262.
Law, L. W., Morrow, A. G., and Greenspan, E. M. 1952. *J. Natl. Cancer Inst.* **12,** 909–916.
Lederberg, J. 1952. *Physiol. Revs.* **32,** 403–430.
Lettré, H. 1950. *Naturwissenschaften* **37,** 335–336.
Levaditi, C. 1914. *Compt. rend. soc. biol.* **77,** 258.
Lewis, M. R. 1937. *Am. J. Cancer* **29,** 510–520.
Lewis, M. R., and Lichtenstein, E. G. 1938. *Growth* **2,** 375–383.
L'Heritier, Ph. 1951. *Cold Spring Harbor Symposia Quant. Biol.* **16,** 99–112.
Little, C. C. 1914. *Science* **40,** 904–906.
Little, C. C. 1934. *J. Exptl. Med.* **59,** 229–250.
Little, C. C. 1947. *Biol. Revs. Cambridge Phil. Soc.* **22,** 315–343.
Little, C. C., and Pearsons, J. 1940. *Am. J. Cancer* **38,** 224–233.
Little, C. C., and Strong, L. C. 1924. *J. Exptl. Zool.* **41,** 93–114.
Little, C. C., and Tyzzer, E. E. 1916. *J. Med. Research* **33,** 393–453.
Loeb, L. 1902. *J. Med. Research* **3,** 44–73.
Loeb, L., and Genther, I. T. 1928. *Proc. Soc. Exptl. Biol. Med.* **25,** 809–811.
Lorenz, E. 1950. *Am. J. Roentgenol. Radium Therapy* **63,** 176–185.
Lorenz, E., Eschenbrenner, A. B., Heston, W. E., and Uphoff, D. 1951. *J. Natl. Cancer Inst.* **11,** 949–967.
Lorenz, E., Heston, W. E., Deringer, M. K., and Eschenbrenner, A. B. 1946. *J. Natl. Cancer Inst.* **6,** 349–353.
Lumsden, T. 1937. *Am. J. Cancer* **31,** 430–440.
Lundback, H., and Løfgren, S. 1950. *Acta Med. Scand.* **138,** 460–470.
Lynch, C. J. 1926. *J. Exptl. Med.* **43,** 339–355.
MacDowell, E. C., Potter, J. S., and Taylor, M. J. 1945. *Cancer Research* **5,** 65–83.
MacDowell, E. C., and Richter, M. N. 1930a. *J. Cancer Research* **14,** 434–439.
MacDowell, E. C., and Richter, M. N. 1930b. *J. Exptl. Med.* **53,** 823.
MacDowell, E. C., and Richter, M. N. 1932. *Biol. Zentr.* **52,** 266–279.
MacDowell, E. C., and Richter, M. N. 1935. *Arch. Pathol.* **20,** 709–724.
MacDowell, E. C., and Taylor, M. J. 1948. *Proc. Soc. Exptl. Biol. Med.* **68,** 571–577.
MacDowell, E. C., and Taylor, M. J., and Broadfoot, J. 1951. *Carnegie Inst. Wash. Yearbook.* **50,** 200–203.
Mann, I. 1949a. *Brit. Med. J.* **2,** 251–253.
Mann, I. 1949b. *Brit. Med. J.* **2,** 253–255.
McEndy, D. P., Boon, M. C., and Furth, J. 1944. *Cancer Research* **4,** 377–383.
Mercier, L. 1937. *Compt. rend soc. biol.* **124,** 403–405.
Miller, F. R., and Turner, D. L. 1943. *Am. J. Med. Sci.* **206,** 146–152.
Morgan, W. C. 1950. *J. Natl. Cancer Inst.* **11,** 263–368.

Mühlbock, O. 1948. *Acta brev. neerl. Physiol.* **16,** 22–27.
Mühlbock, O. 1950. *J. Natl. Cancer Inst.* **10,** 861–864.
Murphy, J. B., and Sturm, E. 1925. *J. Exptl. Med.* **42,** 693–700.
Murray, W. S., and Little, C. C. 1935. *Science* **82,** 228–230.
Murray, W. S., and Little, C. C. 1939. *Am. J. Cancer* **37,** 536–552.
Murray, W. S., and Warner, S. G. 1947. *J. Natl. Cancer Inst.* **7,** 183–187.
Nettleship, A., and Henshaw, P. S. 1943. *J. Natl. Cancer Inst.* **4,** 309–319.
Nicod, J. L. 1936. *Bull. assoc. franc. étude cancer* **25,** 743–755.
Nilsson-Ehle, H. 1909. Kreuzungsuntersuchungen an Hafer und Weizen, Lund.
Rask-Nielsen, R. 1938. *Acta Path. Scand.* **15,** 285.
Richter, M. N., and MacDowell, E. C. 1929. *Proc. Soc. Exptl. Biol. Med.* **26,** 362.
Richter, M. N., and MacDowell, E. C. 1933. *J. Exptl. Med.* **52,** 823.
Schweitzer, M. D., and Furth, J. 1939. *Am. J. Cancer* **37,** 224–230.
Shapiro, J. R., and Kirschbaum, A. 1951. *Cancer Research* **11,** 644–647.
Shimkin, M. B., and Andervont, H. B. 1941. *J. Natl. Cancer Inst.* **1,** 599–605.
Slye, M. 1931. *Am. J. Cancer* **15,** 1361–1386.
Smith, F. W. 1945. *Science* **101,** 279–281.
Smith, F. W., and Strong, L. C. 1949. *J. Natl. Cancer Inst.* **10,** 423–428.
Smith, W. E. 1952. *Arch. Ind. Hyg. and Occupational Med.* **5,** 25–33.
Snell, G. D. 1948. *J. Genetics* **49,** 87–108.
Snell, G. D. 1951a. *J Natl. Cancer Inst.* **11,** 1299–1305.
Snell, G. D. 1951b. *Anat. Rec.* **111,** 84.
Snell, G. D. 1952a. Physiopathology of Tumors. C. V. Mosby Co.
Snell, G. D. 1952b. *Cancer Research* **12,** 543–546.
Snell, G. D. 1953. *J. Natl. Cancer Inst.* (In press.)
Snell, G. D., Borges, P. R. F., Kelton, D., and Allen, S L, 1952. Records of the Genetics Society of America, p. 70.
Snell, G. D., Cloudman, A. M., Failor, E., and Douglas, P. 1946. *J. Natl. Cancer Inst.* **6,** 303–316.
Snell, G. D., and Higgins, G. F. 1951. *Genetics* **36,** 306–310.
Snell, G. D., and Kelton, D. 1953. To appear in *Cancer Research.*
Sonneborn, T. M. 1947. *Quart. Bull. Indiana Univ. Med. Center* **9,** 51–55.
Sonneborn, T. M. 1951. *Cold Spring Harbor Symposia Quant. Biol.* **16,** 483–503.
Spencer, R. R. 1942. *J. Natl. Cancer Inst.* **2,** 317–332.
Spicer, C. C. 1947. *Brit. J. Cancer* **1,** 298–310.
Staff of National Cancer Institute. 1945. Mammary Tumors in Mice. *Publ. Am. Assoc. Advance. Sci.* **22.**
Staff of Roscoe B. Jackson Memorial Laboratory. 1933. *Science* **78,** 465–467.
Stasney, J., Cantarow, A., and Paschkis, K. E. 1950. *Cancer Research* **10,** 775–782.
Stewart, H. L., and Andervont, H. B. 1938. *Arch. Pathol.* **26,** 1009–1022.
Stewart, H. L., Grady, H., and Andervont, H. B. 1947. *J. Natl. Cancer Inst.* **7,** 207–225.
Strong, L. C. 1926a. *J. Exptl. Med.* **39,** 447–456.
Strong, L. C. 1926b. *Genetics* **11,** 294–303.
Strong, L. C. 1926c. *Am. Naturatist* **60,** 201–226.
Strong, L. C. 1929. *J. Cancer Research* **13,** 103–115.
Strong, L. C. 1945. *J. Natl. Cancer Inst.* **5,** 339–362.
Strong, L. C. 1948a. *Science* **108,** 688–689.
Strong, L. C. 1948b. *Cancer* **1,** 120–124.
Strong, L C. 1950a. *Science* **111,** 381–382.
Strong, L. C. 1950b. *Brit. J. Cancer* **4,** 315–320.

Strong, L. C. 1950c. *Yale J. Biol. Med.* **22,** 303–307.
Strong, L. C. 1951. *J. Gerontol.* **6,** 340–357.
Strong, L. C., Hill, R. T., Pfeiffer, C. A., and Gardner, W. U. 1938. *Genetics* **23,** 585–595.
Strong, L. C., and Smith, G. M. 1936. *Am. J. Cancer* **27,** 279–284.
Suntzeff, V., Burns, E. L., Moskop, M., and Loeb. L. 1936. *Am. J. Cancer* **27,** 229–245.
Taylor, M. J., and MacDowell, E. C. 1949. *Cancer Research* **9,** 144–149.
Tyzzer, E. E. 1907. *J. Med. Research* **17,** 137–152.
Tyzzer, E. E. 1909. *J. Med. Research* **21,** 519–528.
Tyzzer, E. E., and Little, C. C. 1916. *J. Cancer Research* **1,** 387–88.
Van Gulik, P., and Korteweg, R. 1940. *Am. J. Cancer* **38,** 506–515.
Wells, H. G., Slye, M., and Holmes, H. F. 1941. *Cancer Research* **1,** 259–261.
Woglom, W. H. 1929. *Cancer Revs.* **4,** 129–214.
Woolley, G. W., Fekete, E., and Little, C. C. 1940. *Proc. Soc. Exptl. Biol. Med.* **45,** 796–798.
Woolley, G. W., Fekete, E., and Little, C. C. 1943. *Science* **97,** 291.
Wright, S. 1934. *Genetics* **19,** 537–551.

The Role of Viruses in the Production of Cancer

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CONTENTS

L'étude des infections est à peine commencée, pouvons-nous nous vanter d'en avoir pénétré tous les mystères?
A. BORREL

It is now more than fifty years since Borrel (1903) founded the hypothesis that cancer has a viral origin on the frail foundations of reasoning by analogy. This hypothesis has had a curious destiny. It was so contrary to everything that was known—or was thought to be known—that from the beginning everyone rallied against it. Nevertheless the facts in its favor have continued to increase slowly but surely, until at the present time the virus hypothesis has become a crucial point in discussions on the etiology of cancer. Whether one likes it or not, it offers the best explanation for malignancy; yet now, as always, it has the united forces of the greater part of cancerologists ranged against it. Where viruses do exist, attempts are made to demonstrate that the transmissible factor cannot be exogenous but that it is produced by the deranged cell. Haddow and some others think that it may thus be possible to reconcile the results of chemically induced cancer with the virus hypothesis and that the "ens malignitatis," to use the term beloved by Teutschlaender (1928), may in fact be a false virus, a cellular particle which has become autonomous. Thus one turns to the organelles of the cell which can become self-reproducing, such as the mitochondria, the microsomes, and the plasmagenes, and the definition of infection, which from the time of Pasteur seemed firmly associated with the idea of an exogenous agent, is now called into question. The discussion thus expands to consider the fundamental problem of the nature and origin of viruses. But before entering into a discussion of such general questions, we shall examine more closely the various virus tumors, and especially the agents that cause them. The account will thus be centered mainly upon the etiological aspect, other questions being treated only in so far as they are indispensible for a knowledge of the properties and mode of action of the blastogenic viruses. As the subject is a vast one, we shall deal mainly with recent results, referring those who wish for information concerning earlier work to the previous reviews by Foulds (1934), Thomsen (1939), Olson (1940),

Engelbreth-Holm (1942), Lépine and Lafforet-Furiet (1943), and Oberling and Guérin (1943).

The virus-induced tumors of birds, amphibia, and mammals will be described in turn, which is in the order of their discovery.

I. Virus-Induced Tumors of Birds

The fowl is a species with an exceptionally high incidence of malignant tumors. Most of these are sarcomas, but dysgerminomas and malignant epitheliomas also occur, often closely resembling those of mammals (Feldmann, 1932; Olson and Bullis, 1942). So far, however, filterable agents have been reported only from sarcomas. It is possible that the epitheliomas are also caused by agents of this type (Oberling and Guérin, 1933), but this has not yet been proved. The etiological study of the epitheliomas is difficult, since they have seldom been transmitted, for, with rare exceptions (Foulds, 1937a; Duran-Reynals, 1946b), attempts to graft them have failed.

1. The Avian Sarcomas

A. *Some Remarks on the Pathology of These Tumors.* The filterable tumors of birds have very diverse structures, and although many resemble the fibromyxosarcoma described by Rous in 1910–1911, others differ from it to a greater or lesser degree and resemble fibrosarcomas, osteochondrosarcomas, polymorphic sarcomas, etc. (see Foulds, 1934, for further details of these). Thus it is certain that these tumors are caused by distinct agents, for even those sarcomas that are morphologically identical, such as the Rous and the Fujinami, can be shown to be caused by viruses that can be clearly distinguished from each other.

The morphological characters of these tumors are not fixed and invariable, for some change to a form similar or identical with that of the Rous sarcoma. More usually, however, they retain a certain degree of structural and developmental individuality and this is maintained even after transmission by filtrates. The virus, in fact, not only causes the malignant transformation of the cells but also confers upon the tumor its individual characteristics. This may be due to the fact that the virus attacks only a certain definite cell type, such as the fibroblast, histiocyte, or endothelial cell, so that the morphogenesis of the virus is nothing more than the consequence of a very narrow cytotropism. But this explanation does not apply to all tumors, for there are tumors that are known to arise from the same type of cell but yet can be clearly differentiated from each other. The most interesting is the osteochondrosarcoma of Tytler. This tumor retained its special character not only when grafted but even when transmitted by filtrates, and Rous, Murphy, and Tytler (1912a,b)

emphasized the fact that this virus, on contact with ordinary connective tissue, produced not an ordinary spindle-cell sarcoma, but a tumor composed of cartilage and later of bone. It is true that this was not maintained, and in later passages the tumor became an ordinary sarcoma composed of undifferentiated cells. Nevertheless, this striking ability of the sarcoma viruses to influence the differentiation of cells should be borne in mind, and it will be discussed again in the general section.

In the study of the various problems concerning the pathology of the avian sarcomas, the Rous sarcoma has usually been employed, chiefly because of the generosity of the Rockefeller Institute in making this material available to research workers throughout the world.

The histogenesis of this tumor is known from the systematic researches of Mauer (1938) and Levine (1939) on the first stages of tumor development after intramuscular injection of filtrates or a suspension of dried tumor tissue. The first reaction is characterized by an accumulation of round cells similar to the monocytes of the blood and local histiocytes, and it is in no way specific; but the presence of the virus is soon made manifest, between the eighteenth and forty-eighth hour, by the continually increasing number of fibroblasts resulting from a transformation of the histiocytic polyblasts which increase very rapidly and soon become the dominant element of the initial tumor nodule. The Rous sarcoma should therefore be considered as a tumor of the histiomonocytic mesenchyme with a predominating development of fibroblasts. This confirms the results previously obtained by Ludford (1932) and Haddow (1933) by the use of vital staining. These researches are also interesting as showing the speed of the malignant transformation, which was completed within 48 hours.

It is natural to seek for something in the cells that indicates the presence of a virus, especially as Sanfelice (1927) had demonstrated the presence of characteristic inclusion bodies in a round-cell sarcoma of the fowl. Nothing of this type has been found in the Rous sarcoma. Eosinophilic granules occur in the nucleus and in the cytoplasm, and Tenenbaum and Doljanski (1943) remarked upon the frequency of intranuclear bodies which resemble inclusion bodies but differ from them by their basophilic reaction. There remain many unsolved problems in connection with the cellular changes produced by viruses, such as the part played by mucoid secretion that is almost always found in avian virus-induced sarcomas (Peacock, 1946). The eosinophilic material that so interested Borrel (1926) indicates very important alterations in the paranuclear part of the cytoplasm, i.e., in the region of the Golgi body and the centrosphere.

The Rous cells have been studied with the electron microscope by

Claude, Porter, and Pickels (1947), by Oberling, Bernhard, Guérin, and Harel (1950), and by Oberling and Bernhard (1952). In the outer regions of the cell, which were the only parts accessible with the techniques so far employed, an abundance of chondriocontes is found, often forming large masses, together with a very great number of vesicular elements corresponding in every way to the microsomes of Claude. The cytoplasmic granules whose abundance had so impressed Borrel (1926) are certainly not due to virus, as he had thought, but represent degenerate mitochodria or more often microsomes enlarged by overstaining.

All modern cytological researches have confirmed the idea generally accepted since the work of Carrel that the Rous cells, far from having a great capacity for growth and exaggerated vitality, are in fact mostly sick cells, and their pure culture, even in the hands of such experts as Carrel and Doljanski and his collaborators, is impossible after a few subcultures (Tenenbaum, 1943). Furthermore, this behavior is shown by all the cellular varieties of the tumor, the fibroblasts as well as the round cells, contrary to the idea previously advanced by Carrel, according to whom only the macrophages were the malignant element. If Carrel was unable to reproduce the Rous sarcoma by pure cultures of fibroblasts originating from this tumor, this was an isolated case and not a general rule. Similarly, the suggestion of Doerr (1937) that only the macrophages could be infected, for they alone could phagocytose the virus, which has no power of active penetration and therefore cannot invade fibroblasts, must be rejected. Ludford (1937) was in fact able to infect pure cultures of fibroblasts derived from the pectoral muscle of fowls with the viruses of Rous and Fujinami. Sandford, Likely, Bryan, and Earle (1952) showed in a detailed study of the behavior of the Rous virus *in vitro* that it maintained its activity longest (over six months) in the presence of fowl fibroblasts and concluded that these represent the essential element upon which it acts.

The main problem which arose as soon as the electron microscope became available has been the hunt for the virus particles in the cells of the filterable sarcomas. In the first investigations to this end undertaken by Claude, Porter, and Pickels (1947), fairly dense spherical particles, isolated or more often forming pairs or short chains, were found in the cytoplasm.

The absence of similar structures in normal cells, together with their size (67 to 84 mμ) which corresponded to that of the virus and their obviously greater density than the microsomes, all led Claude and his collaborators to identify these particles with the causal agent of the sarcoma. The most impressive point perhaps was that in another filterable tumor, "chicken tumor 10," similar particles were found, but other-

wise arranged, forming groups closely resembling clumps of staphylococci. The impression produced by the figures was that of two viruses of the same size but with different arrangements inside the cell, like bacteria in an exudate. Similar appearances were found in later investigations by Bernhard and Oberling (1953), but they were extremely rare, and in hundreds of cells examined by the electron microscope no such particles were found. Many explanations can be offered for this result. It is possible that the tumors studied by Claude *et al.* were exceptional and that usually the virus occurs in the more central parts of the cell that are so far inaccessible to the electron microscope. It is also possible that their tumors were unusually rich in virus and that the number of virus particles in the cell is more often much less. This idea receives considerable support from the quantitative studies of Carr (1947). Taking as a basis the amount of virus obtained by the best methods of extraction, e.g., those of Claude and Rothen, 3.75×10^7 infectious doses per gram of tumor tissue are obtained. If this dose corresponds to one virus particle, the astonishingly low figure of 5 virus particles per 100 cells is obtained, and even if the minimum infectious dose is taken as 1000 particles, the number of virus particles in one cell will not exceed 50, which leaves little hope of demonstrating their presence by the use of the electron microscope. This seems to show that even in such a tumor as the Rous sarcoma most of the virus may be found not in the particulate condition but in the masked state. At any rate, these problems merit much more investigation, for they touch upon a crucial point concerning the whole pathology of virus-induced tumors.

B. *Isolation of the Virus.* The use of ultrafiltration or methods based upon capillary attraction (Bedson and Bland, 1929) allowed a rough separation of the virus in extracts of sarcomas, but they can scarcely be called methods of purification since they did not affect the proteins or other materials to which the virus could be absorbed.

The first attempts at isolating the agent of the Rous sarcoma were undertaken with physical chemical methods used for the purification of enzymes. The use of absorbents such as kaolin, activated charcoal, and alumina gels was the basis of the researches between 1925 and 1935, of which those of Claude (1935) were undoubtedly the most extensive. The methods he developed were subsequently successfully applied by Dmochowski (1948b) to other filterable tumors. The latter gives a complete bibliography of these researches in his work.

Ledingham and Gye (1935) having shown that the agent could be sedimented at least in part by ultracentrifugation, McIntosh (1935) obtained complete sedimentation in 20 to 60 minutes at 40,000 to 60,000*g* in a Henriot-Huguenard type of centrifuge. Using the same principle,

Claude made the most complete concentration of virus so far obtained. After two centrifugations at 17,000*g* for 120 to 165 minutes he obtained a fraction corresponding in dry weight to about 3.5% of the original extract, or 2.2 mg. per gram of tumor. Quantities as small as 5.2×10^{-12} g. produced vigorous tumors after intradermal inoculation into Plymouth Rocks. Taking the weight of a virus particle, calculated for a diameter of 7×10^{-6} and a specific gravity of 1.3, as 2.34×10^{-16} g., this corresponds to about 20,000 particles. Claude suggested that one-tenth of this would have produced tumors if he had carried his dilutions that far.

Dmochowski (1948a,b) confirmed the findings of Claude not only for Rous but also for Fujinami. He found that with the centrifugal forces that he used (15,000*g*) the agent was not completely sedimented, the supernatent remaining active. This perhaps indicates that smaller particles are present as well as those of the usual size—an interesting point that will be returned to later.

Riley (1948, 1950) employed chromatography for purifying the virus, using Celite, a preparation of infusorial earth which strongly absorbs the virus when the salt concentration of the suspending medium is about the same as physiologically normal solutions (0.154 *M*). In passing, it might be noted that this shows the unsuitability of normal saline solutions for work with absorbing filters. Elution was carried out with very weak solutions (0.001 *M*). Starting with extracts already purified by other methods, Riley obtained a concentration of 660/100 in terms of nitrogen content. Calculating the nitrogen content of a single virus particle of 70 mμ diameter as 0.2×10^{-16} g., the minimum infective dose corresponds, according to Riley's calculations, to about 24 particles, or perhaps 15 if impurities are taken into account, and certainly below 100. This represents a considerable reduction from the figure of 2000 calculated by Claude for the minimal infective dose.

All these methods suffer from the disadvantage of being applicable only to small amounts of tumor tissue and produce virus in an unstable form. Immunological and biochemical research requires much greater quantities capable of being preserved in an active condition for long periods. Carr and Harris (1951b) set out systematically to solve this problem. Using a Sharples centrifuge, together with tryptic digestion which removed 60% of the nitrogenous material without affecting the infectivity of about a million infective doses per gram of tumor, the final product contained about 1.7 to 2% of the nitrogen of the original extract. This product, suspended in Lemco broth and lyophilized, preserved its activity for a year or more (Carr and Harris, 1951b).

C. *Properties of the Virus.* From the investigations upon the size of the virus, using graduated collodion filters (Elford and Andrewes, 1935;

Yaoi and Nakahara, 1935) or centrifuging (McIntosh, 1935), a size of 70 mμ is obtained. The values obtained by the electron microscope are more debatable, because of the difficulty of distinguishing the virus from some tissue components, especially the microsomes. Riley (1950) obtained interesting results with virus purified by chromatography, finding many particles below the usually accepted size of 70 mμ. Those fractions possessing the greatest activity contained particles varying between 20 and 70 mμ, the majority being about 40 mμ in size. The results of Dmochowski (1948b) which suggest a similar result have already been discussed, and the findings of Pentimalli (1948) may be mentioned in this context. Emphasizing the constant absorption of the virus upon proteins, he concludes that all previously established sedimentation constants are invalid and that the sarcoma agent may well have a smaller size than has been so far admitted. Thus the actual size of the Rous virus is not yet definitely established. All that can be stated is that the diameter of 70 mμ seems to be a maximum and that particles of this size may already be an aggregated form of the virus. This seems the more likely, since Bryan, Lorenz, and Moloney (1950) showed by treating the virus with x-rays that the dimensions of the active particle corresponded to about 48 mμ.

In the purified condition the virus is extremely fragile, but adsorption upon proteins confers a remarkable resistance. The results so far described relating to the resistance to various physical and chemical agents refer usually to the adsorbed form and not to pure virus. The apparently unlimited resistance to desiccation, or to glycerol in which it loses only 50% of its activity in a month, or to radiations, may be mentioned in this connection. According to Lacassagne and Nyka (1938), six million roentgens are required to inactivate the virus, although the sarcoma tissue is killed by very much less, in fact the 1000 to 6000 r usually employed in the treatment of tumors (Miszurski, Pikowski, Goldhaber, and Doljanski, 1945).

It is interesting in this connection to refer to the excellent experiments of Peacock (1946) on the behavior of these sarcomas treated with suitably chosen doses of 4000 to 10,000 r. The tumor vanished, but the virus was not inactivated. It was resorbed by the lymphatics, entered the general circulation, and became fixed a certain distance around the zone of irradiation, where it gave rise to an outburst of sarcomatous nodules. This localization undoubtedly corresponded to a position where the reaction of these tissues to the radiation was particularly favorable for the fixation of the virus.

The virus is readily destroyed by ordinary antiseptics, bile, saponin, or most dyes. If treated with antibiotics *in vitro* before inoculation, an inhibition results (Chinn, 1952). Aminopterin seems to affect the virus,

for chicks fed a diet containing this material show retardation of the tumor growth (Ringsted, 1952). The virus is very resistant to cold and remains active for long periods if kept at −70° (Epstein, 1951, 1952). On the other hand, it is very sensitive to heat, being destroyed in 15 minutes at 56° and rapidly inactivated at 37°.

D. *Immunology of the Avian Sarcomas.* Although there have been very many researches relating to the immunology of the avian sarcomas, it must be confessed that the results are often contradictory and deceptive. There are many reasons for this. First, the antigens used were not pure. As generally employed, the preparation of sarcoma virus always contained a certain amount of fowl protein. The methods of purification were just beginning to be studied, and meanwhile each author used a different method for the preparation of his antigens. Furthermore, these antigens were not titrable. The only possible method, that of determining the minimal infective dose, depends upon the receptivity of the fowl, which is very variable, as will be shown later. This is particularly troublesome, as the content of virus of the tumors is extremely variable.

Moreover, the virus is very fragile; its instability increases with the degree of purification attained, and it loses its activity rapidly at 37°. This is important in results depending upon the neutralization of the virus, and even with methods based upon complement deviation the result may depend upon the activity of the virus (Dmochowski, 1948a). To this must be added another difficulty, the presence of the Forssman antigen in the normal tissues and tumors of the fowl (Witebsky, 1929; Gereb and Simke, 1932; Dmochowski, 1938). It should also be noted that the significance of serological tests depends very much upon the nature of the antigen-antibody reaction being studied; flocculation, precipitation, and agglutination are usually more marked with species-specific antigens than with viruses. Furthermore, tests employing the inactivation of virus in the presence of complement often lack specificity because of the presence of species-specific antigens. Probably the best reactions are those that use the neutralization of the virus after prolonged contact in the cold in the absence of complement.

Finally, the fowl is one of the least suitable animals for serological researches: first, because of the anticomplementary activity of its serum; next, and above all, because of the frequency of isoantibodies as demonstrated by Landsteiner and Miller (1924), Todd (1930), and later workers. If the serum of any fowl will react with the red cells of any other fowl, then it is obvious that there are probably antibodies in it that will react with extracts of various tissues, as was effectively demonstrated by Duran-Reynals (1940a).

(a) *Natural immunity.* Carrel first noted (1925) that the fowl con-

tained natural antibodies to the Rous virus, and that the content of these "physiological antibodies" increased with age. This was confirmed by many others, including Andrewes (1931, 1933b, 1936), Yoshikawa and Ishimota (1930), Rous, McMaster, and Hudack (1935), Ledingham and Gye (1935), Amies (1937), Amies and Carr (1939), and Duran-Reynals (1940b). Only in the percentage of animals showing this resistance do the results show any considerable variation. Such natural neutralizing antibodies are found not only in adult or aged animals but also sometimes in young ones. Andrewes (1939) showed that the serum of young chicks and even the egg yolk could contain them if the maternal blood had a high titer. This resistance of maternal origin was also noted by Duran-Reynals (1940b). It is lost some time after hatching, but the antibodies of the animal itself can appear after the sixth week (Carr and Harris, 1951a), so that at all ages the fowl may contain materials antagonizing not only the Rous virus but all sarcoma-producing agents. The fowl is therefore the worst animal to use for titrating its own sarcomas.

In a detailed study of the antagonistic materials in normal blood, Duran-Reynals (1940b) showed that they belonged to the globulin fraction which usually contains antibodies. The amount diminishes when the bird bears a sarcoma, probably as a result of being fixed by virus entering the circulation. On the other hand, it is not affected by chemical tumors. In general, they seem to be true antibodies and distinct from the isoantibodies which cause flocculation with normal tissues or sarcomas. No relation was found between the amount of tissue-extract-flocculating substance and virus-neutralizing power of a given serum.

Neutralizing substances are also found in the duck, not only for the Rous virus but also for its duck-adapted variants, and they can be detected five hours after hatching, thereafter increasing rapidly in amount (Duran-Reynals, 1943a). This is rather surprising, since it concerns an experimental tumor which does not exist naturally. It is difficult, therefore, to attribute such natural immunity to minute amounts of antibody resulting from subclinical or inapparent infections. Perhaps there are some natural antigens related to the sarcoma viruses which the fowl absorbs and thereby immunizes itself. Heredity also plays a part, as was shown by Greenwood, Blyth, and Carr (1948), who selected a line of fowls particularly resistant to the Rous virus. The whole problem is certainly complicated, and suggesting a "serological maturation" to explain them, as some have done, rather implies that this term was invented merely to hide our ignorance. Above all, it is essential to avoid regarding the appearance of the natural antibodies as indicating some relationship between these viruses and the normal constituents of the cells that they may infect. It should be sufficient to recall that, for ex-

ample, the guinea pig regularly contains agglutinins against the gonococcus, and that horses and cattle may have antibodies against the cholera vibrio in regions where cholera has never been encountered within living memory (Doerr, 1949).

(b) *Acquired immunity.* A high level of antibody is always found in the blood of a fowl bearing a slowly growing or regressing tumor, or after such a tumor has been absorbed (Andrewes, 1931, 1933b). Also, injection of Rous viruses into various species (ducks, geese, goats, rabbits) will produce antisera which neutralize or flocculate the virus (Rous, Robertson, and Oliver, 1919; Muller, 1931; Gye and Purdy, 1931; Murphy, Sturm, Favelli, Hoffman, and Claude, 1932).

This much established, there were two problems that especially interested investigators. First, they were interested in determining the serological relationships of the various viruses of the filterable tumors. For this, the pheasant proved a particularly useful animal, as it can be infected by most of the fowl tumors and belongs to the same Forssman group as the fowl, thus permitting a more refined analysis. Andrewes (1933b) was thus able to show that the Fujinami virus was neutralized only by its own antiserum, although an anti-Fujinami serum readily neutralized the Rous and many other sarcoma viruses. The Rous virus was closely related to a whole group which were neutralized by anti-Rous serum. There was a third group, MH2, RF4, which occupied an intermediate position, for their antisera neutralized neither Rous nor Fujinami. Within each group, each virus could be differentiated by qualitative reactions, and Andrewes concluded that all the sarcoma viruses of the fowl are related, but that no two are identical.

The second problem was to see if these sarcoma agents could be differentiated from the species-specific proteins. Here the results have been complicated, sometimes contradictory, and on the whole disconcerting. Andrewes first had the idea of using the Fujinami sarcoma for this research, for this tumor has the useful property of growing in the duck as well as in the fowl. The duck never contains normal antibodies against Fujinami, but after regression of a Fujinami tumor the blood contains a high level of antibodies. These neutralized not only duck Fujinami but also fowl Fujinami and several other sarcoma viruses (Andrewes, 1931). It can therefore be concluded that the sarcoma viruses contain something in common but differ from the species-specific antigens. This was confirmed by the later proof by Andrewes (1933b) that fowls could not be immunized against duck Fujinami by injecting duck embryo. All the same, Purdy (1933) succeeded in immunizing ducklings against fowl Fujinami by injecting fowl embryos. The indications are less certain with the pheasant, which after treatment with fowl embryos shows some

resistance to Rous virus, but much less than after injection of sarcoma extract (Andrewes, 1933a).

Amies (1937) then showed that rabbit antifowl sera produced by injecting citrated blood were capable of neutralizing the sarcoma viruses, and saw thereby a fundamental difference between these agents and the ordinary viruses such as vaccinia. Unfortunately the value of these results is much reduced by using the rabbit for these experiments, for because of the Forssman antigen it does not lend itself readily to such work. These findings of Amies, amplified later by Amies and Carr (1939), do not invalidate the earlier and contrary results of Gye and Purdy (1931, 1933) and Gye (1936), who used the goat to produce antiserum.

Dmochowski (1948a) employed the complement fixation test, using as antigen Rous and Fujinami viruses highly purified by ultracentrifugation. Such viruses did not deviate complement with rabbit antifowl serum, although the reaction was strongly positive with an antifiltrate serum. These results seem to indicate a clear differentiation between the sarcoma agents and the species-specific proteins. Unfortunately they are not free from criticism, for the antifowl sera were produced from plasma, whereas according to Gye the common antigen between the sarcoma viruses and normal tissues is probably nuclear in type. Furthermore, Dmochowski himself admits that these results might have been different if the antifowl sera had been more powerful, which again indicates the inconvenience of serological reactions where, for technical reasons, the quantitative factor cannot be taken into account.

It thus seems clear that the viruses of the avian sarcomas contain two types of antigens, one being virus proper, the other of fowl origin. The amount of the latter may vary with the degree of purification, and it may well be that the most complete purification has not yet eliminated all species-specific antigen. Whatever criticism may be made of Dmochowski's results, they mark such progress that a future separation of the two antigens may be discounted and will not depend merely upon perfecting methods of extracting the virus. Anyhow, since other viruses such as influenza and equine encephalitis also frequently contain tissue material, any differences between the antigenic make-up of the avian sarcoma viruses and that of other viruses are only of degree.

E. *Infection by the Virus.* We shall discuss in turn infection by tumor virus of the adult fowl, the chick, the fowl embryo, and finally infection of other species (heteroinoculation).

(a) *Infection of the adult fowl.* Most of the facts concerning infection of the adult fowl were established by the earlier investigators and need not be recounted here. Only a few points which are still the subject of debate will be mentioned.

(1) Conditions for Tumor-Producing Activity. In general, the virus will produce a tumor only when it penetrates into the connective tissue. If injected into the blood, it fails to produce a tumor if all contact with connective tissue can be avoided. Tumors are, however, produced if kieselguhr is injected together with the filtrate (Rous, Murphy, and Tytler, 1912a,b) or if some lesion is produced in the animal which will permit the virus to reach connective tissue. If special precautions are not taken, a tumor will often form at the site of injection and thus falsify many experiments upon infection by the intravenous route.

That virus in the circulating blood will not produce a tumor is in itself astonishing. It must be accepted that the monocytes of the blood, the reticuloendothelial cells of the marrow and the spleen, and the Kupffer cells of the liver, which it must inevitably encounter, are all insensitive to it. It thus seems that the sarcoma-inducing action of the virus is evident only in the special conditions found in injured cells. It should be remembered that in the blood the agent is highly diluted and immediately adsorbed by the corpuscles or proteins. In fact, it is easy to produce tumors from such animals by injecting whole blood, erythrocytes, leucocytes, plasma, or fibrin, but filtrates of blood rarely produce anything. The virus is thus rapidly fixed but not destroyed, and when injected into tissues in this condition it is probably liberated from these combinations by the action of proteolytic enzymes.

(2) Temporary Disappearance of Filterability of Tumors. Everyone who has worked with the virus tumors of fowls has noted at some time or other that there are periods when the transmission by filtrate becomes difficult or impossible. Many authors since Rous and Murphy have drawn attention to this sort of occurrence (see the review of Foulds, 1934, p. 6). This absence of filterability often occurs together with a marked reduction in the growth rate of the tumor, and it may last for several months, when the virulence and filterability suddenly reappear. Fraenkel (1930) claimed that this loss was seasonal, filtrates being usually more active in spring than in winter. The same thing has been observed at this Institute and has been the object of systematic studies by Vigier. During these periods the other methods of transmission of filterable tumors such as grafts of desiccated tissue, glycerination, or repeated freezing and thawing are equally negative. This may be related to the observations of Peacock on the chemically induced tumors of the fowl (1935a, 1946). For three consecutive years the tumors had a marked reduction in virulence during the period of July to January, so much so that some lines were lost by failures of their grafts. Murphy and Sturm (1941a) have made similar observations.

It seems to us that the most probable reason for these periods of non-

filterability is a reduction in the amount of free virus in the tumors. It occurs when the tumor is inoculated into an environment unsuitable for the spread of the virus, such as old hosts, abundant inhibitor, high antibody level, or lack of certain vitamins or hormones. This last may explain the seasonal effect. The most striking argument in favor of this is supplied by the contrary results; by avoiding such conditions as may interfere with the production of virus, as was done by Claude, tumors are obtained which remain filterable for years and always contain large quantities of virus. It is not impossible that in certain cases the tumors become nonfilterable because the virus enters into a masked phase, like the bacteriophage in certain lysogenic strains. This recalls the experiments of Miszurski, Pikowski, Goldhaber, and Doljanski (1945), who were able to transmit three out of five nonfilterable tumors after irradiation with 5000 r, despite the destruction of some tumor tissue. If these experiments could be repeated with absolute certainty that cellular transmission was excluded, they would present a striking analogy with the very recent researches where active bacteriophage was liberated from lysogenic strains after treatment with ultraviolet radiation.

(3) Filterability of Spontaneous Tumors. Nebenzahl (1934) reviewed about thirty spontaneous tumors examined in various parts of the world and concluded that all sarcomas which could be transmitted for a sufficient time became filterable, sometimes from the beginning (Gye, 1936), more usually after a few or many passages. Duran-Reynals (1946a) studied fourteen spontaneous sarcomas of which nine could be transmitted for at least one passage. Only five of these could be continued. These last were all found to be filterable, one from the beginning, the others after further passage.

The adversaries of the virus theory often point to the small percentage of spontaneous tumors that finally become filterable. But this is a distortion of the facts. What is really low is the percentage of spontaneous tumors that become transplantable (four out of forty observed at the Rockefeller Institute by Murphy and Claude, 1933). This, as everyone is aware, is partly due to other reasons, for the transplantations are often done in very unfavorable conditions. What is important, on the contrary, is that *all* sarcomas that have been successfully transplanted have sooner or later become filterable, and this was recognized by Murphy and Sturm, who stated (1941b) that "all tumors of the latter class (i.e., spontaneous) if properly investigated, have given evidence of containing a transmissible agent."

(4) Filterability of Chemically Induced Tumors. The same conclusion seemed to apply to the chemically induced tumors of the fowl whose study was begun by Carrel (1925). These tumors were found to be trans-

missible by filtrates, but similar studies undertaken in other laboratories (Murphy and Landsteiner, 1925; Sturm and Murphy, 1928; Kauffmann, 1929; Felloni, 1930; Laclau and Pillado-Matheu, 1930; Mellanby, 1934) failed to confirm Carrel's results. Only White (1927) succeeded in producing a sarcoma by injecting minced 9-day-old embryo mixed with 1/50,000 arsenious acid, and this tumor appeared after the fifteenth day and was transmissible by filtrate after a single passage by graft.

Subsequently the problem was taken up again by McIntosh (1933), who obtained thirteen tumors from twenty-one hens injected with tar, three of which could be transmitted by filtering through a Berkefeld V. In 1936, another tumor became filterable after three years of transmission by grafts. In 1939, in collaboration with Selbie, he presented four further observations upon tar tumors of which two were transmissible by filtrates. They remarked upon a clear relation between filterability and increase in growth rate.

In the same period, Peacock (1933, 1936) reported on some very extensive researches bearing upon this point, and during work extending over fifteen years obtained fifty-five tumors by treatment with tar or dibenzanthracene. Of this large series of tumors, all spindle-celled sarcomas, thirteen were successfully transplanted, one for thirty-one passages, but not one was transmissible by filtrate. After a critical survey of McIntosh's tumors—justifiable, perhaps, but in our opinion not final upon some points such as tumor No. 9—Peacock concluded (1946) that transmissibility by filtrates of the chemically induced tumors does not occur, that these tumors can be separated from the filterable sarcomas by a series of morphological and biological characters, and that in all cases where filterability of a chemically induced tumor was claimed there were technical errors. To this he ascribes a positive result he himself obtained.

It is, however, possible that in certain cases the tumors may have been contaminated by Rous virus present in the laboratory. Begg and Cramer (1929) described such an error occurring during their experiments, and we consider that this possibility should be considered on each occasion that a tumor, considered as chemically induced, develops very rapidly and shows itself to be filterable immediately. This probably applies to the tumors of Carrel and perhaps also to that of Des Ligneris (1935), which was obtained *in vitro* by keeping a culture of fowl fibroblasts in contact with dibenzanthracene. According to Amies, Carr, and Purdy (1939) this tumor cannot be distinguished from Fujinami either histologically or by cross-immunity tests. Mellanby (1938) studied these possibilities experimentally by grafting Rous sarcomas to fowls bearing chemically induced tumors, when they were found to contain virus in the same amounts as the liver, spleen, and other organs. Carr (1946) showed

that chemical tumors infected artificially by virus from a grafted tumor contained only minimal quantities of Rous virus, insufficient to induce the formation of anti-Rous antibodies when grafted into another animal.

Nevertheless, these experiments show the possibility of infecting chemical tumors with ordinary sarcoma viruses, and Hadfield and Garrod (1948) were certainly right in saying that the subject should be reconsidered and that no account should be taken of results obtained in laboratories where other viruses are used. Oberling and Guérin (1950) described a sarcoma that appeared in the foot of a hen thirty-two months after injection of an oily solution of methylcholanthrene. This tumor, which grew slowly, could be transmitted by grafts, and attempts at transmission by filtrate were negative at the second and third passages. At the fourth passage grafts made with glycerinated material were negative, but a month later, in the following passage, positive results were obtained with fragments kept for 7 to 27 days in glycerol in the cold. At the fifth passage, twenty months after the appearance of the primary tumor, transmission by filtrate was positive, as it was also at the sixth passage. It should be mentioned, however, that an attempt at filtration at the seventh passage was negative, as if the virus had returned to a masked form. It is thus clearly established that at one part of its history the tumor became filterable; the gradual changes in its behavior were indicated by resistance to glycerol and, later, by filterability. Furthermore, this observation is especially interesting because infection in the laboratory can be completely discounted, since, because of the restrictions due to the war, no filterable tumor was maintained in the laboratory during the period that this filterability manifested itself.

In conclusion, it is interesting to mention certain immunological facts which similarly seem to suggest the existence of a virus in the chemically induced tumors, even though they have not yet been transmitted by filtrates.

Using a nonfilterable fowl tumor produced by dibenzanthracene, Foulds (1937b) injected filtrates or tumor mince into rabbits and thus produced antibodies which neutralized Rous virus. These were active in the absence of complement and were not adsorbed by normal fowl tissues. They thus resembled anti-Rous antibodies in all respects, but it must be confessed that these and the subsequent reports of Foulds and Dmochowski (1939) are not in our view definite proof of the presence of a virus in the chemically induced tumor. It should be remembered that anti-Rous antibodies may be evoked by inoculation of fowl tissue not containing virus (Amies, 1937; Amies and Carr, 1939).

Dmochowski and Knox (1939) showed that a filtrate of a sarcoma induced by dibenzanthracene would induce antibodies in rabbits to give a

positive complement fixation test with filtrates of Rous, MH2, and duck Fujinami. This last finding eliminates certain errors contained in the previous work. But it has not yet been shown that the common antigen in the filterable tumors and in chemically induced ones really corresponds to the virus. It may well be some other antigen, such as the Forssman, for example. Therefore in our opinion the only experiment of conclusive value is that of Andrewes (1936). He succeeded in transmitting a tar-induced nonfilterable sarcoma of Mellanby to pheasants and showed that neutralizing antibodies for Rous appeared in their blood. The important point was that the chemically induced tumor was transmitted to another species, for Andrewes was careful to show by precipitating reactions that the tumors developing from these grafts were really pheasant tumors and not heterografts of fowl tumors. This can be explained only by the presence of a sarcoma-inducing virus.

To conclude this very controversial section on chemically induced tumors, we can only remark that these tumors do not at the outset resemble the filterable sarcomas. They are usually more compact, more fibrous, slowly growing, and without the myxomatous areas so characteristic of the filterable sarcomas. But this holds also for many spontaneous tumors. And we have seen that these tumors, which are usually nonfilterable, may suddenly become so if they are transplanted for long enough. These are therefore caused by filterable agents which reveal themselves only in certain ill-defined conditions which arrive by chance during passage. The same may be said of the chemically induced tumors. They are not filterable, but they may become so. Irrefutable evidence exists to prove this.

(b) *Infection of the young chick.* When injected intravenously into young chicks, the virus produces a hemorrhagic disease that kills the animals within 12 days (Duran-Reynals, 1940c). At autopsy numerous hemorrhages—petechiae, hematomas, or blood cysts—are found on the surface of the viscera, especially the liver, spleen, and peritoneum, and also in the lungs, kidney, skin, heart, muscle, periosteum, and pericardium. Sometimes the hemorrhagic lesions are mixed with sarcomatous nodules, especially in the liver and spleen. In the young fowl this combination of the two processes becomes more frequent, and as the animal ages the hemorrhagic aspect decreases in favor of the tumorous. The formation of these hemorrhagic lesions depends upon many factors, such as the age of the host, the virulence of the agent, and the amount of virus injected. Similar lesions have been produced with Fujinami virus, but not with the MH2 endothelioma. They are also to be found in ducklings injected with Rous virus adapted to the duck. In these circumstances Duran-Reynals (1950) even found cerebral hemorrhages related to

vascular lesions characterized by endothelial proliferation and consequent necrosis of the vascular walls.

It is natural to inquire whether these lesions may not be due to an associated virus. Duran-Reynals produced the following facts against such an interpretation: (1) Injection of organs containing hemorrhagic lesions into adult birds invariably reproduced the sarcoma, even after multiple passages in the very young chick. The Rous sarcoma can therefore be transmitted in two different forms, either as the hemorrhagic disease in young chicks or as a sarcoma in the adults. (2) Transitions between the hemorrhagic lesions and sarcomas are often found, especially in rather older chicks. (3) The lesion can be prevented by the use of serum of older birds (Duran-Reynals and Estrada, 1940).

(c) *Infection of the embryo.* Murphy and Rous (1912) were the first to inoculate embryonated eggs with tumor material, and they thereby inaugurated a method that was to prove extremely valuable in the future. They showed that the Rous sarcoma could be successfully grown in various parts of the embryo and its membranes, producing tumors on the chorioallantoic or allantoic membranes or in the embryo itself. Embryos of all ages were susceptible, although the most suitable times were between the seventh and tenth days. Tumors were also obtained with filtrates or desiccates. Furthermore they successfully inoculated pigeon and duck eggs, although the resulting tumors could not be transplanted to adults of these species, even after prolonged passage upon their eggs. To these fundamental results the workers who took up the subject again twenty years later had little to add. Oshima and Yabuuchi (1933) inoculated hen, duck, and quail eggs, readily obtaining tumors on the membranes without causing metastases in the embryo, although the tissues and organs contained active virus even after hatching. Milford and Duran-Reynals (1943) injected extracts or filtrates into embryos of various ages by the intracoelomic or intravenous route. They obtained no tumors, only hemorrhagic lesions identical with those seen in the young chick. In only two cases did tumors appear on the chorioallantoic membrane together with hemorrhagic lesions in the embryo. The special interest of this work resides in the fact that they were able to demonstrate the existence of necrotic lesions in the walls of the vascular system and surround tissues that were not without analogy to those produced by ordinary viruses such as those herpes.

More recently, Bryan, Kahler, and Riley (1945) cultivated the Rous sarcoma in the yolk sac; and Karnofsky, Parisette, Patterson, and Jacquez (1949) studied the effect of x-rays and nitrogen mustard on sarcomas grown on the chorioallantoic membrane. Oker-Blom (1951), working in the laboratories of Duran-Reynals with the tumors studied by

him (ordinary Rous and the duck-adapted 14(D)7) attempted to determine the conditions which cause the hemorrhagic disease in fowl embryos. Inoculation of minced cells of the sarcomas on the chorioallantois produced tumors there, usually together with hemorrhagic lesions of the membrane and the embryo. A tumor together with hemorrhagic lesions of the embryo was also found in ducks. Filtrates injected intravenously or intraembryonically caused the hemorrhagic disease only.

However, in researches not yet published, Vigier could not produce the hemorrhagic disease by injection of large doses of filtrate into either newly hatched chicks or 12-day-old embryos. Only embryos bearing large tumors in the yolk sac showed the typical hemorrhagic disease. It therefore seems likely that the strain of fowls used has something to do with the production of this phenomenon.

(d) *Heteroinoculation.* Many experiments have been performed to determine the susceptibility of other species to the filterable tumors of the fowl, and these are of interest, since they touch upon the fundamental problem of the mutation of the virus. For a long time, the Rous virus could not be successfully transmitted to other animals, although Fujinami would grow in ducks (Fujinami and Suzue, 1928). The Rous sarcoma can be transplanted to the anterior chamber of the guinea pig eye by the technique of Greene, and after growing there it contains a virus which when injected into fowls will produce periosteal tumors, which the ordinary Rous virus never does (Shrigley, Greene, and Duran-Reynals, 1945). Andrewes (1932, 1933a) succeeded in transmitting to the pheasant not only Rous by both cells and filtrates, but also many other sarcomas such as Fujinami, MH2 and 2BS1. Des Ligneris (1932) transmitted Rous to the turkey and guinea fowl, but only by cells; filtrates produced no result. Duran-Reynals (1943b) showed that this transmission by filtrates was possible if young animals were used, for with the guinea fowl aged up to 5 weeks or the turkey aged up to 6 weeks about 80% produced sarcomas after injection of filtrate. The resulting tumors were slow fibrosarcomas producing massive metastatic growths in the liver and spleen of the guinea fowls and the osteoperiosteal tissue in the turkey. After passage in the latter, a distinct change in behavior was noted. It became active in older animals, and if transferred back to fowls it produced osteoperiosteal tumors. In this connection it is interesting to note that this can be produced with the Rous virus by prolonged conservation (Shrigley, Greene, and Duran-Reynals, 1945) or by prolonged culture *in vitro* (Pikowski and Doljanski, 1946).

The most interesting researches in connection with the heterotransplantation of Rous sarcoma were made by Duran-Reynals upon the duck. It was known for a long while that the Rous could not be trans-

mitted to the duck, but by injecting large doses of filtrates into newly hatched ducks Duran-Reynals (1942) obtained lesions in about 22% of the animals. These were of two types: lesions which appeared within the first month after injection, and retarded lesions appearing only after several months when the animals were adults. The early tumors could be retransmitted to fowls without difficulty, although inoculation into other ducks was always without result. Their causative virus had thus retained its fowl character. The late tumors could be transmitted only to very young chicks, although they could be transferred to other ducks without difficulty. The period in the duck had therefore transferred the virus into a duck virus, which on passage gave rise to various tumors—fibrosarcomas, osteosarcomas, giant-cell sarcomas, angiomas, lymphosarcomas, and leukemias.

These tumors behaved to the fowl in the same way that the Rous sarcoma behaved with ducks. They seldom produced tumors in adults, and these always regressed. Only inoculation into very young chicks gave frequent takes, and these also gave immediate and late tumors. The immediate tumors developed in about three months and gave rise to spindle-cell sarcomas and periosteal proliferation. The late lesions were leukemias or osteopetrosis.

The study of these tumors is of extreme interest, since the virus acquires a certain lability of cytotropism and biological behavior and can give rise to very diverse lesions. Further varieties obtained by using the duck were later described by Duran-Reynals (1947). In some cases the virus became adapted to the duck but retained its affinity for the adult fowl, in which, however, it no longer produced Rous sarcomas but different forms of tumor with localization in the bones. One strain lost its duck affinity and became a fowl virus again, but the passage through the duck had induced a profound modification which made it pathogenic for the pigeon of any age. It may be added that in the pigeon it occurred in a masked condition, for Borges and Duran-Reynals (1952) failed to transmit it to either the duck or fowl, although after passage by cell graft through the fowl it became filterable again.

2. Transmissible Leucoses of Birds

The fowl leucoses were the first neoplastic disease in which viruses were shown beyond all doubt to play an etiological role. But when this fundamental fact was proved by Ellermann and Bang in 1908 it was not suspected that the avian leucoses and tumors would one day pose one of the greatest problems in the etiology of cancer, and their discovery did not receive the attention it merited.

The avian leucoses are of two types, erythroblastosis and lymphomatosis, which for several reasons need to be studied separately.

A. *Erythroblastosis.* Four main forms of erythroblastosis can be distinguished according to the type of abnormal cells in the blood: pure erythroblastic leukemias, hemocytoblastic erythroleukemias, erythroleukemias with a myeloid reaction, and pure myeloid leukemias.

There is nothing much to add to the detailed descriptions of the clinical and morbid anatomy of these conditions given in the works mentioned at the beginning of this review. Engelbreth-Holm and Rothe-Meyer (1932b) first showed that the young chick was especially susceptible and that the disease was more rapid in such animals. Engelbreth-Holm also (1935) indicated the importance of the seasonal factor, as shown by the fact that inoculations gave 80% takes in April and May, but only 40% in October and November. Such seasonal variations were not found with very young animals. It is important to remember that avian erythroblastosis, unlike the mammalian leukemias, is not always fatal; it is not unusual to find that animals with undoubted clinical signs of the disease, and whose blood can transmit the condition to other fowls, may yet recover. Sometimes the only manifestation of the disease is a transitory alteration in the hematological picture. Leucosis can therefore exist as a latent disease, and only systematic examination of the blood will reveal its course.

(a) *Properties of the leukemia virus.* The agent of the transmissible leucoses occurs in the blood, ascitic fluid, and organ extracts, and is separated by filtration of such material by Berkefeld and Chamberland filters, as Ellermann and Bang (1908) showed. Furth and Miller (1932) showed by filtration through collodion membranes that the size was comparable with that of bacteriophage.

As with all virus diseases, the fluids containing the agent are active at extreme dilutions. Furth (1932) showed that 10^{-5} ml. of blood or 10^{-6} ml. of plasma would transmit the condition, as we can confirm. Eckert, Beard, and Beard (1951) found that inoculation of blood gives quantitatively different results from inoculation with filtered plasma, showing a shorter latent period, a higher incidence of takes, and a greater infectivity. It is interesting that the relation between the amount of plasma injected and the latent period or percentage of takes should be linear, since for other viruses (fowl sarcomas, rabbit papilloma, etc.) it takes the form of a sigmoid curve (Eckert, Beard, and Beard, 1951). There is, however, no relation between the number of particles or of primitive cells and the infectivity (Eckert, Sharp, Beard, and Beard, 1952). Most of the virus in the blood is adsorbed by the red cells, but even in plasma or

serum, like other viruses, it is attached to proteins, especially the globulins (Jármai, 1938).

Purification of the virus was attempted by Beard, Eckert, Csaky, Sharp, and Beard (1950), who filtered heparinized plasma of leukemic fowls through 0.2 Selas filters (Selas Corporation of America, Philadelphia), centrifuged the filtrate for 60 minutes at 20,000*g*, resuspended the deposit in Simm's solution, clarified by slow-speed centrifugation, and recentrifuged twice at 15,000*g* for 30 minutes. The sediment showed spherical particles of average size 60 to 100 mμ, having, like some bacteriophages, a tail about 100 to 200 mμ long. Since such elements were completely absent from normal plasma or the plasma of fowls resistant to leukemia, they considered that they probably represented the agent of the leucosis. In work recently published, Sharp, Eckert, Beard, and Beard (1952) found similar spermatozoal-like forms in the blood of leukemic fowls, but consider that in normal conditions the virus has the form of a sphere of about 120 mμ diameter.

The erythroblastosis virus is more resistant to heat than the avian sarcoma viruses. Minced organs or leukemic blood kept at 37° will lose their activity in 15 days. It is usually destroyed by heating to 57° for 30 minutes, although using the OG strain Greppin (1937) obtained positive results after heating for 40 minutes. It is very resistant to cold, withstanding the temperature of liquid air, and is equally resistant to freeze-drying. Pieces of fowl liver dried by Plotz in the Flosdorf-Mudd apparatus were active after one and one-half years. It is extremely resistant to x-rays (Jármai, Stenszky, and Farkas, 1932; Engelbreth-Holm and Rothe-Meyer, 1932a). Blood or pieces of tissue retain their activity for three months in glycerol (Furth, 1932; Oberling and Guérin, 1934a). On the other hand, it is rapidly inactivated by oxygen, but can be reactivated by reduction (Engelbreth-Holm and Frederiksen, 1938; Ruffilli, 1938a).

(b) *Attempt at culture in vitro.* Following the first attempts of Verne, Oberling, and Guérin (1936), who were able to maintain the virus in an active state in cultures of fowl bone marrow for 15 days, Furth and Breedis (1937) had better results using a virus of less restricted cytotropism, which could induce proliferation in endothelial cells and fibroblasts as well as in blood cells. Virus 13, which thus can give rise to both sarcomas and leukemias, could be maintained in cultures of sarcoma cells for 150 days, and when re-inoculated into fowls again produced both sarcomas and leukemias. On the other hand, if a nonsarcomatogenic erythroblastic virus is cultivated with cells from another sarcoma, it soon vanishes. This suggests that a cyto-stimulating virus multiplies only in the presence of cells that it can itself induce to multiply. It was therefore

surprising when Ruffilli (1938b) succeeded in maintaining the OG virus for 122 days simply by cultivating the myocardium of a leukemic fowl. This was confirmed by Doljanski and Pikowski (1942), who used the myocardium of a normal fowl explanted in leucotic plasma and obtained leukemias after culture for a period of four weeks. They also succeeded in conserving the virus for 178 days in cultures of fibroblasts.

(c) *Growth of the virus in the fowl egg.* Injection of blood or minced organs of leukemic fowls into the embryonated egg will usually produce a leukemia which kills the chick, especially if the inoculation is made after the tenth day, when the bone marrow appears (Jármai, 1933; van den Berghe and d'Ursel, 1939). However, success has been obtained occasionally with earlier inoculations (Kirschbaum, Stern, and Hooker, 1940). Atanasiu, Vieuchange, and Strunge (1951) maintained leukemia on the embryo for eleven successive passages, and in such material Atanasiu (1952) found myeloid leukemia in 1.08% of cases. Kirschbaum, Stern, and Hooker (1940) succeeded in producing leukemias by injection of filtrates, although filtrates are usually without result (Vigier, 1951). Such filtrates remain infectious for about 10 days (Pierce, 1942).

Vigier and Guérin (1951) found that serial inoculation of leukemic marrow on the chorioallantoic membrane of 7- to 13-day-old embryos not only produced typical leukemias but also small erythroblastomas on the membranes, one of which was associated with sarcomatous proliferation, and that these could be reproduced either by direct grafting or by intramuscular inoculation of the blood or bone marrow of the hosts. Vigier has shown that the virus will remain active after at least 16 days in the yolk sac.

(d) *Behavior of the virus in susceptible animals.* Like all other viruses, the agent of avian leucosis has a special affinity for certain types of cells. This cytotropism is especially well shown after intravenous injection. It soon disappears from the blood and is retained by the immature blood cells of the erythroblastic lineage in the bone marrow. It is probable that it is in these cells that it multiplies while inciting them to abnormal proliferation, and it can be found again in the circulation at the end of 5 to 6 days, and thereafter occurs in all organs and tissues of the body.

If it thus acts as a cyto-stimulating virus like the sarcoma viruses but with a hemotropic affinity, it is interesting to determine whether it can incite proliferation in other cells and thus cause malignant tumors. This can in fact be done, and the first experimental demonstration of the sarcomatogenic action of the leukemia virus was provided by Oberling and Guérin in 1933. By inoculating fowls with pieces of organs or leukemic blood that had been preserved in glycerol in the cold for some time, they obtained sarcomas of various types, some giving metastases. Grafts of

such sarcomas produced leukemias again, and this cycle of leukemia–sarcoma–leukemia could be repeated. Altogether 80 tumors were produced in 128 passages. In two cases epithelial proliferations were found in close contact with the sarcoma, producing an appearance resembling certain human epitheliomas. The origin of this epithelioma was in one case a cyst caused by traumatic inclusion of a piece of skin; in the other, the surrounding epithelium. Although small, these epithelial proliferations are of theoretical importance, as they indicate that the leukemic virus can act not only upon mesenchyme but also upon epithelial cells. In certain cases the remote tumors were morphologically the same as these at the injection site, but others were quite different, including fibrosarcoma of the kidney, endothelioma of the pericardial serosa, reticulosarcoma of the bone marrow, and myosarcoma of the intestine. Obviously the virus was exhibiting a marked instability of its cytotropism, shown not only by the formation of such diverse tumors but also by proliferations of the vascular endothelium and reticuloendotheliomas of the liver, spleen, and marrow resulting in something looking like a true reticulosis (Oberling and Guérin, 1934b). Intradermal inoculations frequently gave rise to peculiar granulomas showing fibroblastic and histiocytic proliferation and containing large numbers of eosinophiles (Oberling, 1937). The affinity of the virus can thus extend to various mesodermal derivatives, producing a whole series of cancers that can be considered as "mesodermoses." These experiments demonstrate modifications of the cytotropism of the leukemic virus, the controlling mechanism of which, despite considerable research, remains unknown. This sarcomatogenic action of leukemia virus was later confirmed by Rothe-Meyer and Engelbreth-Holm (1933), Jármai (1935a), Storti and Zaietta (1938), and Troisier (1934). Rothe-Meyer and Engelbreth-Holm (1933) observed a particularly interesting strain which regularly produced leukemias by intravenous inoculation and sarcomas after intramuscular injection.

Furth alone (1935) for some time disputed this conception of a sarcomatogenic action of leukemic virus, considering it to result from the interaction of two viruses, one hemotropic, the other histiotropic. But the result of his own researches, especially those with Breedis (Furth and Breedis, 1937) on the growth *in vitro* led him to adopt the unitary hypothesis, and finally to offer the most convincing proofs.

Direct proof of the sarcomatogenic action of the virus was provided for the OG strain by Greppin (1937), who obtained a tumor at the site of injection of a Chamberland filtrate; this sarcoma, growing in an animal free from indications of leukemia, was grafted to ten fowls, nine of which died of leukemia and in six of which a small tumor was also found. Subsequent careful histological studies by Storti and Zaietta (1938) showed

that the fibrosarcomas produced by virus arose from the connective tissue cells present at the site of inoculation. However, the induction of sarcomas by a direct action of the virus upon connective tissue was denied by Pikowski, Goldhaber, and Doljanski (1947). Employing strain T of Engelbreth-Holm, they showed that intramuscular inoculation of cell-free material such as ultracentrifuged plasma or liver irradiated with 50,000 r had no such action. Sarcomas arose, however, if whole blood or liver containing living leukemic cells was used, which seemed to suggest that the tumors arose from hemocytoblasts which anyhow are known to be capable of transforming into fibroblasts *in vitro*. Such tumors, moreover, did not develop like true sarcomas but remained small, never metastasizing and incapable of transmission by grafting. Subsequently Pikowski and Doljanski (1950) formulated their point of view more precisely, postulating the existence of two sorts of erythroblastic viruses, simple and combined. The former never produce tumors after inoculation of virus alone, the tumors observed being in reality fibroblasts arising from transformed hemocytoblasts of the graft, and not true sarcomas. The combined virus can produce true tumors by reacting directly with the fibroblasts of the injected animal.

The results of Doljanski and his collaborators are interesting and important, but in our opinion they are not sufficient to establish such a clear-cut difference into two groups of leukemic virus as they claim. The same strain may at one period produce only false sarcomas by grafts of hemocytoblasts and later sarcomas by inoculation of filtrates or filtered plasma. Anyhow, the strain that they used produced tumors with filtered plasma in the hands of Uhl, Engelbreth-Holm, and Rothe-Meyer (1936). Furthermore, the term "combined" should be rejected, as it may lead to confusion with "mixed virus," although as used by Pikowski and Doljanski it was applied to a single virus having a double tropism, hemotropic and histiotropic.

It thus seems established that under certain circumstances the leukemic viruses can modify their cytotropism and produce sarcomas. But the ability to effect this change is inconstant and varies from strain to strain. The essentially neoplastic nature of the leukemic process is established, however, and the old conception of Bard that leukemia is a cancer of the blood has received a brilliant experimental confirmation from this modern work.

Jármai (1935b) succeeded in transplanting the erythroblastosis to pheasants, guinea fowls, and turkeys. Passage in the latter produced a marked diminution of virulence for the fowl. Engelbreth-Holm and Rothe-Meyer (1932a) also showed that it could be transmitted to the guinea fowl, and Oberling and Guérin (1943) succeeded in transmitting

the leukemia to guinea fowls and turkeys, the best results following intracerebral injection of leucotic blood. Serial passage through guinea fowls was not possible, however. The virus of fowl leucosis is thus only pathogenic for a few avian species, and even in these its activity is rapidly reduced. This is the more remarkable since spontaneous leukemias are not peculiar to fowls but have probably been noted in most birds. They have been reported in ducks and pigeons (Lund, 1927; Forestier, 1933), in turkeys (Reinhardt, 1930; Jármai, 1930), in budgereegahs (Reinhardt, 1930), in parrots and storks (Fox, 1923), and in canaries (Haupt, 1928; Farkas, 1930; Satterlee, 1906; Kögler, 1933). An erythroleukemia has been transmitted for one passage in the latter animal (Schoenaers, 1946).

It must be admitted that each species has its own leukemic viruses, and since for each species each strain has its own special behavior, one concludes that there must be a surprising number of viruses, a conception that was also reached for the filtrable tumors.

(e) *Immunology.* The problems of immunity to the erythroblastoses of fowls have been the object of many researches, the main points of which will be mentioned here.

(1) Natural Immunity. Some animals are refractory to the leukemic virus, but this immunity is not always complete and it is not unusual for animals to die after re-inoculation although they may have withstood several previous injections.

(2) Acquired Immunity. This is shown in animals which after inoculation have become carriers of this disease. It should be noted, however, that the diagnosis of transitory or abortive forms of the disease is difficult or often impossible, so that the distinction between natural and acquired immunity is not always easy to make. The demonstration of specific antibodies by complement fixation or precipitation has not so far been successful (Greppin, 1937; Kabat and Furth, 1940). Animals that have acquired immunity to leukemia usually have neutralizing antibodies in their blood. These are not present in the blood of species that are insusceptible to the virus (duck, pigeon) but appear after the injection of leukemic blood. Engelbreth-Holm, Rothe-Meyer, and Uhl (1935) showed that these antibodies are not related to the antifowl factors, since the serum of ducks treated with normal fowl blood failed to neutralize the leukemic virus. The antibodies not only can neutralize the virus present in plasma but can even inhibit the agent bound to leukemic cells.

These antibodies are not altogether specific, and they can be induced by certain avian tumors. The resistance produced by tumor extracts is complete if the tumor is derived from a sarcoma of the same strain (Guérin and Guérin, 1947), and this suggested trials with formalinized

blood as a vaccination against fowl leukemia (Guérin, 1948). The many attempts to induce active or passive immunity to the erythroblastoses in fowls have not so far succeeded (Oberling and Guérin, 1934a, 1943; Greppin, 1937; Guérin and Guérin, 1947; Engelbreth-Holm, Rothe-Meyer, and Uhl, 1935).

(f) *Natural transmission.* The means whereby the disease is spread remains uncertain. Since minute quantities (1/100,000 ml.) of blood are sufficient to transmit it to another fowl, it is natural to think of insects as vectors of the virus. But except for a single positive result by Ellermann (described by Anderson and Bang, 1928) with the bedbug (*Cimex lectularius*), all such attempts using *Argas* (Jármai, 1932), fowl lice (Wakamatzu, 1934), and *Dermanyssus* (Anderson and Bang, 1928) have been without result, and the manner by which it is naturally disseminated is as yet unknown.

B. *The Lymphomatoses.* If in many ways the lymphomatoses have been overshadowed by the erythroblastoses in the fowl, this is due solely to the fact that the former were not susceptible to transmission, and this of course made experimental investigation impossible. Actually, lymphomatoses are much more common than erythroblastoses, and in certain countries where the production of pure breeds of fowls is very common the condition may become a veritable plague, killing up to 30.6% (Winton, 1948) and even 60% (Olson, 1948) of the animals in certain poultry farms. The Government of the United States, disturbed by the annual losses ($75,000,000, according to Cottral, 1951) created experimental stations for the study of the disease and to find means to combat it. Of these, that at East Lansing (Michigan) has acquired a world-wide renown for the quality of its work.

As Ellermann first showed, the lymphomatoses usually cause an aleukemic or extravascular disease. The four recognized forms are visceral, ocular, neural, and skeletal.

The visceral form, one of the commonest, causes single or multiple tumors affecting the liver, lungs, pancreas, ovary, muscles, skin, etc. Whatever organ or tissue may be affected, it is not unusual to find also diffuse infiltration of a leukemic nature, or sometimes an involvement of the blood which clearly indicates the leukemic nature of the malady.

The ocular form shows itself by a grayish coloration of the iris caused by leukemic infiltrations, which spreads to cause protusion of the eyeball, whose increasing opacity recalls the appearance of the eyes of cooked fish.

The neural form was described as neurolymphomatosis by Pappenheimer, Dunn, Cone, and Seidlin (1929). Clinically it takes the form of multiple paralysis, involvement of the wings or legs being the most com-

mon and characteristic. Involvement of other areas may result in dyspnea or diarrhea. This form produces epizootics of great severity, decimating entire flocks. It shows a special affinity for highly bred Leghorns, i.e., flocks subject to considerable selection (Lhermitte, de Ajuriaguerra, and Souquet, 1943). The neural involvement is not always pathognomic of lymphomatosis, for Guérin and Guérin (1949) observed it with erythroblastic leukemia.

Finally, the lymphomatosis may cause skeletal changes of the type referred to as osteopetrosis, where the diaphyses of the long bones are attacked and they show proliferation of the spongy bone both at the periphery and in the interior. The cancerous bone then develops a spongy structure not unlike a meringue, and the medullary cavity becomes progressively reduced.

(a) *Transmission.* From the outset the transmission of the lymphomatoses proved much more difficult than the erythroblastoses. The earliest transmission of neurolymphomatosis was by Pappenheimer, Dunn, and Cone (1929) and by Furth and Breedis (1935). But the results of these researches are rendered dubious by the fact that they were obtained with strains in which the disease already existed in enzootic form, and it was not until 1941 that the first transmission of the tumorous form of lymphomatosis was obtained, by Pentimalli (1941) with a lymphosarcoma, and by Olson (1941) with a lymphoid tumor of the bursa of Fabricius. This latter was grafted for thirty passages with 68% takes and 44% regressions. Brewer and Brownstein (1946) succeeded in grafting a visceral lymphomatosis for fifteen passages with 59% takes.

The demonstration of a filterable agent was made by Burmester, Prickett, and Belding (1946). Using a strain grafted for 200 passages, they injected the supernatants of centrifuged extracts of tumors or filtered plasma of tumor-bearing animals into 2-day-old chicks. No local tumor resulted, but the disease appeared after several months, frequently in the form of a combination of visceral lymphomatosis and osteopetrosis. Later Burmester and Denington (1947) and Burmester and Cottral (1947) isolated ten new transplantable strains, four of which could be transmitted by filtrates. The agent of the lymphomatosis must be present in large amounts in the blood, for filtered plasma was as effective as filtrates from tumors. Intraperitoneal injections were as effective as intravenous ones. The frequency of osteopetrosis was always greater when filtrates were used, and animals developing osteopetrosis contained in their blood an agent which would produce either visceral lymphomatosis or osteopetrosis.

From the experimental results that we have just reviewed, one derives a distinct impression that one and the same virus can produce any form of lymphomatosis, and this led to the idea that all these diseases could be

united under the name of lymphomatosis. This conception was upheld by many authors, especially in so far as it concerned the etiological identity of visceral lymphomatosis and neurolymphomatosis (Bayon, 1931; Gibbs, 1934; Jungherr, 1937; Furth, 1935; Potel, 1938). Davis, Doyle, Walkey, and Cenker (1947) showed that neurolymphomatosis is very frequent in White Leghorns and rare in Barred Plymouth Rocks, whereas visceral lymphomatosis shows the reverse distribution. They concluded that two distinct diseases existed, due to two different agents. As far as osteopetrosis goes, we have already mentioned its special behavior; it is very rare in spontaneous lymphomatosis but especially frequent in transmission by filtrate. In considering the single or multiple nature of the viruses of lymphomatosis, it should be remembered that with the avian sarcomas similar tumors are often due to viruses that can be readily distinguished from each other, and of the agents so far isolated, no two are alike. Therefore it seems very surprising that it should be otherwise for the group of diseases so diverse in type as the lymphomatoses. It can scarcely be doubted that there are many viruses of lymphomatosis, and it becomes easy to regard the various manifestations of the condition as due to different viruses, each characterized by varying cytotropism and probably other factors as well. It is equally certain that local factors have a great deal to do with the determination of the site of action of the virus and thus with the pathological nature of the disease.

Waters (1947, 1951) showed that inherited factors play a part in lymphomatosis and that its appearance depends upon the age of exposure to the infection, the duration of the exposure, and the genetical constitution. In addition, it is possible to vary the frequency of lymphomatosis in certain lines by rigorous selection. Furthermore, genetic selection can influence the type of the lymphomatosis—visceral, neural, or ocular—and lines can be produced having a greater incidence of one form than another, corresponding to the specific susceptibility of the various organs (Waters and Prickett, 1946).

Hormonal factors also play a part, for lymphomatosis is more frequent in hens (30%) than in cocks (9.1%) or capons (14%). Burmester also (1945) showed that lymphomatosis was twice as frequent in males as in females. Treatment with diethylstilbestrol or testosterone propionate showed that the male hormone increased resistance. These hormones did not affect the incidence of the disease in young chicks exposed to infection, estrogen merely reducing the period of incubation (Davis, Andrews, and Doyle, 1950). These hormonal effects may also explain the influence of the age of the animal upon the transplantability of the tumor; in 2-day-old chicks inoculation results in 95% takes, but in 114–day-old pullets it gives only 31% (Burmester, 1952).

Heterografts of lymphomatosis have so far failed. Ducklings, pheasants, guinea fowls, or turkeys have all resisted inoculation.

(b) *Isolation of the virus.* Using methods similar to those employed for the isolation of the erythroblastosis virus, Sharp, Eckert, Burmester, and Beard (1952) showed that the virus of avian lymphomatosis is about 70 to 160 mμ in diameter and possesses a tail.

The virus remains active after storing at −76° for 403 days, but there is a certain amount of loss after lyophilization (Burmester, 1952). Dextrose has a protective action upon cells preserved at −70° (Darcel, 1952). The activity of fresh material is destroyed by heating for half an hour between 50° and 95°. It is destroyed by formalin and ultraviolet radiation, although it will withstand 12,050 r of x-rays (Burmester, 1952).

(c) *Immunity.* After regression of the Olson strain, the birds are resistant to further inoculation. This immunity lasts for about seven months and is strong enough to enable the animal to resist an inoculation of ten thousand times the previous dose (Burmester and Prickett, 1944). Olson (1945a) confirmed this, showing that the immunizing power increased with the number of passages, reaching a maximum after the thirty-sixth passage. No animal resistant to the first inoculation was susceptible on reinoculation, and even the animal bearing a tumor became resistant after the seventh day. Olson also indicated several other interesting features. Freezing inhibited the proliferative activity before the immunizing activity. But the amount of attenuation needed to retain the immunizing activity varied among the tumors. Successive freezing and thawing was more harmful than prolonged cooling (Olson, 1945b). After inactivation by this method the lymphoid tumor is no longer capable of affecting the incidence of spontaneous lymphosarcomas (Olson, 1948). The supernatant after centrifugation, if inactive, cannot induce immunization (Olson, 1945c). The proliferative activity is reduced by heating to 70° for 15 minutes, but it is inhibited if the time is prolonged, and the immunizing power is then lost as well. The tumor is inactivated by desiccation, spontaneous necrosis, phenol, and formol, and then loses its immunizing ability (Olson, 1946).

(d) *Natural transmission.* Our knowledge of this is due mainly to the researches of the U.S. Regional Poultry Research Station at East Lansing (Michigan). The first important discovery at this center was the transmission of the lymphomatosis virus by the egg. In laboratories built to prevent all exogenous contamination, a large flock of fowls was raised from eggs whose shells had been carefully sterilized. The efficiency of these measures was indicated by the fact that all parasitic infections except coccidosis were absent. Lymphomatosis appeared in these fowls after the fortieth day, killing 12.13% in the first ten months and 21.9% by the end

of twenty months (Waters, 1945; Kamarow, 1948; Warren, 1951). Apart from this clear demonstration, later repeated, other facts corroborate this transmission of virus by the egg.

First may be mentioned the existence of lymphoid areas disseminated throughout the tissues of the fowl, whose presence was previously considered normal. Actually, the work of Lucas (1946, 1949), Lucas and Oakberg (1950), and Lucas and Breitmayer (1950) showed that these areas often show invasive tendencies, especially in the pancreas and peripheral nerves, and their increase in size and number seems a reliable indication of the predisposition to lymphomatosis. Everything seems to point to their presence being related to the presence of the virus. These areas are already present in the tissues of the embryo and the newly hatched chick. Second, inoculation of blood or liver, of newly hatched chicks, or of filtrates of embryos from mothers that were clinically free from the infection will sometimes transmit the disease, thus directly demonstrating the presence of the virus in the eggs. This also shows the fact, one of fundamental importance, that an apparently normal hen can harbor the virus and transmit it to her offspring.

Chicks that are thus born carriers of the virus may not die of lymphomatosis, especially if they do not come into contact with highly infected or sick birds. Some will die of lymphomatosis, however, even when completely isolated, which again proves that transmission can occur through the egg. Many will not become ill but may live to pass the virus to their offspring once more, so that lymphomatosis may be transmitted in a latent manner from generation to generation, appearing suddenly one day like a spontaneous disease (Gross, 1951).

This transmission through the egg is not the only mode of transmission. When chicks from a slightly infected source are mixed with heavily contaminated animals, a large number go down with the disease. Infection may therefore take place by contact, and Waters and Bywaters (1949) showed that this took place as aerial infection. Only animals less than 40 days old are susceptible to this route of infection.

The transmission of lymphomatosis thus reduces to a problem of epidemiology which can be dealt with by the classical means used to combat infectious diseases, that is, isolating families and protecting them from all contact with others, and then selecting for breeding purposes those which show the lowest incidence of lymphomatosis. Lines can thus be obtained in which the disease is practically nonexistent. The reverse procedure will give lines wherein the mortality may pass 40 to 50%, but this cannot be readily increased further, as the pullets often die before coming into lay. Elimination can thus be effected by isolation from infection rather than by breeding for resistance. But the latter method

has also been used, and by this means Hutt and Cole (1947) were able to isolate two resistant lines, C and K, and a very susceptible line of Leghorns, which thus allows a genetical study of the disease to be carried out.

The main outline of the spread of lymphomatosis can be understood from these data. Long known, it was held in check in the Old World by the methods of production which usually take place in small flocks of mixed breeds. In the United States the rationalization of the industry led to the development of enormous incubators and rearing stations containing huge numbers of purebred animals. This supplied ideal conditions for the propagation of the virus, so that lymphomatosis, practically unknown in the United States before 1917, has now become a veritable epizootic.

In so far as virus-induced tumors are concerned, these facts about leucosis are extremely instructive, for they concern a disease which exactly resembled spontaneous tumors for forty years in showing sporadic cases refractory to all attempts at transmission, even by grafting. Environmental change was enough to change this condition, which in every way resembled human tumors and leukemias, into an epizootic disease having all the characters of an infection, and thus revealing its essential nature.

II. Tumors of Cold-Blooded Animals

Baldwin Lucké (1934a,b) discovered and investigated a transplantable kidney tumor of the leopard frog (*Rana pipiens*), a glandular epithelioma usually in the form of an infiltrating adenocarcinoma which may metastasize, more rarely a simple adenoma. The frequent occurrence of inclusion bodies in the nucleus suggested that a virus might be concerned in its production. Grafts of pieces of tumor under the skin or into the peritoneum or brain did not cause local tumors, but kidney tumors appeared in about 20% of the frogs after about six months. The same results were obtained using tumor tissue killed by cold, by conservation in glycerol, or by desiccation.

Large tumors often metastasize, usually through the blood stream, which explains their frequency in liver and pancreas, but they may also localize in the bladder, intestine, ovary, peritoneum, and exceptionally in the lungs (Lucké, 1938a,b). The tumor tissue itself shows reduced catalase activity and reduced phosphatase activity, as is often the case in cancer (Lucké, 1952).

The growth of the tumors is influenced by the environmental temperature. High temperatures favor growth and tend to change the histological type to a cystic form; but even at 4° the kidney tumors are

able to grow, which explains their occurrence immediately after the animal has been hibernating (Lucké and Schlumberger, 1940b, 1949).

The kidney tumors could be transplanted into tadpoles. Tail grafts regressed before the disappearance of the tail in the normal course of metamorphosis, but subcutaneous grafts would persist (Briggs, 1942). This resorption during metamorphosis was found in thyroidectomized or hypophysectomized animals (Briggs and Grant, 1943).

Schlumberger and Lucké (1949) succeeded in maintaining serial passage with grafts in the anterior chamber of the eye, which had the advantage of allowing direct examination of the tumor and thus permitting them to follow the changes in growth due to temperature or season. At the first passage, only one tumor appeared in ten animals, but by the fourth passage 100% takes were obtained. The invasive power increased during passage, and some tumors produced liver metastases.

Such intraocular grafts provide excellent material for the study of the action of x-rays in single or divided doses. A single dose of 6000 r results in complete disappearance of the graft, but a single dose of 2000 r causes a transitory regression followed four weeks later by rapid growth. On the other hand, 3200 r in divided doses had no effect, and 7000 to 12,000 r are needed to induce regression. This experiment confirms two known facts of radiology: that greater doses are needed when treatment is divided, and that an insufficient treatment may be followed by an abnormally rapid development of the tumor (Lucké and Schlumberger, 1950a,b).

Heterografts into the anterior chamber of the eye were tried with other species of frogs, toads, and other cold-blooded animals (goldfish, alligators). Some successes were obtained with toads and some species of frogs (Schlumberger and Lucké, 1949) but less readily with more remote species (Lucké and Schlumberger, 1940a).

Occasionally the tumor agent can grow in young salamanders or in the regeneration cells of adult salamanders. When returned to the frog after this passage in foreign tissues, it shows an affinity for skeletal tissues and the iris. Rose and Rose (1952) suggest that the virus may combine with certain cellular constituents by a mechanism comparable with genetical "crossing-over" to produce new forms of virus with altered affinity for tissues.

Another tumor agent was discovered by Lyell Thomas in the Wisconsin frogs. This induced a tumor in the fat body, which could always be successfully transplanted into the anterior chamber of the eye. Some animals developed lipoid tumors in their subcutaneous tissues or in their viscera. One of these was successfully transplanted into the eye and began to lose its adipose character, transforming into a malignant growth at the

third passage; later the liver, mesentery, and ovary showed tumors resembling a small-celled sarcoma (Rose, 1952).

Champy and Champy (1935) described a transmissible epithelioma of the skin in *Triton alpestris* which was probably caused by a virus, but the conditions for transmission could not be specified.

To end this section on cancers of cold-blooded animals, certain neoplasms of fish may be mentioned. Apart from some papillomas of the epidermis and lips and a lymphosarcoma of the gills, there is a lymphocystic disease which attacks both fresh-water and marine fish, such as pike and perch. It produces nodular or flat tumors formed from fibroblasts transformed into giant spherical or ovoid lymphocystic cells. Inclusion bodies are contained in their cytoplasm, which resemble invertebrate eggs, containing osmiophilic granules mostly uniformly about 375 mμ in size (Weissenberg, 1949, 1951; Nigrelli, 1952). Although their number increases during the growth of the inclusion body, it is uncertain whether this is due to division or to a progressive differentiation from the basophilic material of the inclusion body. When this inclusion body ruptures at a later stage, the granules spread through the cytoplasm and are freed by disintegration of the tumor cells, and probably represent the form by which the infection is spread to other animals. Weissenberg (1951) showed that the agent could pass through a Chamberland L5 filter. Nigrelli (1952) called attention to the possible relations of ectoparasites such as ergasalids, other copepods, and mites in spreading the condition.

III. Tumors of Mammals

1. Shope Papilloma

There is nothing much to add to the now classic description of this tumor by Shope (1933) and the detailed studies of Rous and Beard (1934) and Beard and Rous (1934), as regards its behavior in the domestic rabbit. The outstanding thing is the malignant transformation which occurs between the fourth and seventh month in about 75% of domestic rabbits (Rous and Beard, 1935). Preliminary work had suggested that cancerization was exceptional in the cottontail rabbit, no cancerous transformation having been seen in hundreds of trapped animals (Rous, Kidd, and Beard, 1936). After the description of isolated cases of cancers arising in cottontails (Syverton and Berry, 1935; Rous, Kidd, and Beard, 1936), this attitude was changed by the work of Syverton and his collaborators, Dascomb, Koomen, Wells, and Berry (1950). They based their work, not on animals captured by chance, but on papilloma-bearing rabbits kept under observation, and they found that, of 127 observed for over six

months, 32 developed 106 epidermoid carcinomas, with metastases in 19 animals. Cancer thus appears in about 25% of papilloma-bearing wild animals.

The main problem for discussion concerns the behavior of the virus in the cottontail and domestic rabbits, especially as regards its position concerning the cancerization of the papillomas, which will now be considered.

A. *Isolation and Physical Characters.* From data derived from ultracentrifugation experiments, Schlesinger and Andrewes (1937) assigned a size of 40 mμ and a density of 1.3 to 1.4 to the virus. In the same year, Beard and Wyckoff, and then Beard, Bryan, and Wyckoff (1939), isolated a heavy protein by repeated centrifugation of papilloma extracts which produced papillomas when diluted to an amount corresponding to 10^{-7} to 10^{-8} g. The amount of infectious protein depended upon the initial virulence of the tumor and was of the order of 100 to 200 mg. per kilogram of tumor tissue. It contained at least eighteen amino acids, especially glutamic acid (Knight, 1950). Since solutions of this material do not show birefringence of flow, Lauffer and Stanley (1938) concluded that it was spherical in shape. This was confirmed by Sharp, Taylor, and Beard (1942), who showed that in electron microscope pictures the virus was in the form of a sphere about 44 mμ in diameter. Studying this point later by shadowing techniques, Sharp, Taylor, Hook, and Beard (1946) decided that the images were really flattened spheres, but this may be an artifact produced by drying. Later work by Kahler and Lloyd (1952) assigned a diameter of 40 mμ to the particles after metallization, and each particle contained several denser regions about 8 mμ in diameter.

Shope first drew attention to the remarkable thermoresistance of the virus. It can withstand 65° for 35 minutes and is attenuated by heating to 67°, but it requires 70° for inactivation. It is similarly very resistant to ultraviolet light (Kidd, 1938a). This is undoubtedly related to the desert climate of the places where the papilloma is particularly widespread. It can thus retain its activity for some time when shed in the warty masses that detach from the papillomas. The warrens become contaminated, and as the rabbits scratch themselves on entering they inoculate themselves with virus.

It is remarkably resistant to the action of glycerol; preserved in glycerol diluted 50% with water or saline, it may remain active for 14 years, according to Syverton and his collaborators. With regard to x-rays, the high resistance of the virus contrasts with the extreme sensitivity of the papillomas. A single dose of 3000 r (Lacassagne, 1936) or 3600 r (Syverton, Harvey, Berry, and Warren, 1941) will produce a permanent regression of the tumors, although the virus requires very much larger

doses indeed. The actual amount depends upon the degree of purification (Friedewald and Anderson, 1940); nonpurified virus adsorbed to protein complexes requires several million roentgens (twenty-five million, according to Lacassagne, three or four million according to Friedewald and Anderson), although the latter found that only four to six hundred thousand were needed to inactivate virus purified by differential centrifugation.

B. *Susceptible Tissues.* The Shope virus has a cytotropism limited strictly to the epidermis. Large inoculations into all other organs and tissues is always without effect, and even the mucous membranes are insensitive to its action. If a long scarification passing from the epidermis to the mucous membrane is inoculated with the virus, the production of papillomas stops abruptly at the beginning of the mucous layer. Even if the mucous layer is keratinized by avitaminosis A, it remains insusceptible (Kidd and Parsons, 1936).

From the results of Rous, Beard, and Kidd (1936) the embryo skin was regarded as insensitive, but Fischer and Syverton (1951) obtained positive results by inoculating embryos *in utero* intraepidermally. Embryonic skin is therefore susceptible to the virus, but not to the same extent as adult skin. This is true for the virus deriving from a cottontail, but it is much more susceptible than adult skin for virus deriving from the domestic rabbit (Greene, 1953).

C. *Factors Influencing the Development of the Lesions.* The virus must reach the basal cell layer of the epidermis to become effective. The degree of infection, or, in other words, the number of particles reaching a specified region, has a decisive effect not only upon the development of the lesions, their number, extent, and confluence, but also the time of incubation (Syverton and Berry, 1935).

The virus is without effect upon healthy skin and has no effect when injected intravenously (Shope, 1933), although some reactions (trauma, scratches, pyogenic or parasitic infections, burns, etc.) may enable it to reach the basal cells (Rondoni and Eisen, 1937). Subcutaneous injections, even of very large doses, cause tumors only where the needle penetrates the skin. Among the factors which prevent the appearance of the papillomas may be mentioned weak irradiation of the skin, which will induce a localized insusceptibility which lasts for several weeks (Lacassagne, 1937). Hypophysectomy also has an inhibiting action (Lacassagne and Nyka, 1937), although the most obvious antagonistic action is seen with the antibodies (neutralizing antibodies) which prevent extension of the lesion by contamination of adjoining cells in wild rabbits.

Many factors influence the fixation of the virus by susceptible cells and the development of the papillomas, and their effects are interesting

in several connections. Rous and Beard (1934) showed that intradermal inoculations of a saturated solution of Scharlach R in olive oil would favor the infection by the virus, which then produces tumors more rapidly than usual and which, from the outset, extends all over the hyperplastic area thus induced. Furthermore, under these conditions the same result is obtained whether the virus is applied to the skin surface or injected intravenously.

Kidd and Rous (1938), then Rous and Kidd (1938), demonstrated the stimulating action of tar on the infection and growth of the virus. Intravenous injections induce a massive outburst of papillomas on tarred skin, which grow more rapidly and become malignant much earlier, the resulting cancers being also more malignant and more anaplastic than usual. Benzopyrene has a similar effect (Lacassagne and Nyka, 1937). When the virus and chemical carcinogen (methylcholanthrene or 9,10-dimethyl-1,2-benzanthracene) are applied simultaneously, the papillomas appear later, then suddenly become malignant at several points at once at a stage when this seldom happens with ordinary papillomas (Rogers and Rous, 1951).

Friedewald (1942) indicated that preparation of the skin by tar or carcinogenic hydrocarbon could be replaced by painting with a mixture of equal parts of acetone and terebenthine applied five times at intervals of two days. This treatment prepares the site in some way: the basal cells show increased multiplication and the epidermis is obviously thickened. It must precede inoculation of the virus to be effective; applied simultaneously or afterward it shows no action. A single application is also insufficient, at least three being required to produce an effect.

Friedewald later (1944) perfected methods of inoculation. Noting that the scarified skin dried rapidly and that much of the virus was retained by the scab and lost when this detached, he covered the site by a layer of paraffin gauze. This gave an increased yield of about 100 to 1000 times that previously found, and considerably reduced the time of incubation. The minimal infective dose (50% takes) established by Neurath, Cooper, Sharp, Taylor, Beard, and Beard (1941) at 56.8×10^6 virus particles was reduced to 5600 and in some experiments to 2000, which approaches that found for other viruses.

D. *Behavior of the Virus in the Tumors It Induces.* The virus is usually present in the papillomas of the cottontail rabbit, although in varying amounts, being abundant in relatively small papillomas, but it may be absent in the extensive masses formed by confluence of the proliferating papillomatous surfaces (Kidd, 1938c).

In the domestic rabbit the virus no longer occurs free, and these papillomas cannot be transmitted by filtrates (Shope, 1933). The presence

of the virus is shown, however, by the appearance of antibodies in the animal bearing the papillomas which will neutralize the virus of cottontail papillomas and give a complement fixation reaction with this as antigen (Kidd, 1937, 1938b). These antibodies appear about three weeks after inoculation and further increase as the papillomas develop, and more are formed in domestic rabbits than in wild rabbits bearing tumors of equal size. It is difficult to produce antibodies by intraperitoneal injection of papilloma tissue, even after many inoculations, and injection of filtrate is nearly always without result. Furthermore, papilloma extracts will not fix complement when an antivirus serum is used. The papilloma of the domestic rabbit has therefore only a weak and incomplete antigenic capacity. Kidd (1939, 1940a) endeavored to explain this special behavior of the papilloma virus in domestic rabbits by invoking the action of antibody traversing the (frequently swollen) vessels of the stroma of the lesions and thus neutralizing the virus. Some papillomas show a very high content of virucidal antibodies, but this is unlikely to be the explanation of the disappearance of the virus. Shope (1935) showed that certain strains could be maintained in rabbits, and that one and the same animal could bear two sets of papillomas, the one strain being transmissible whereas the other was not. Selbie, Robinson, and Shope (1948), and Selbie and Robinson (1948) obtained strains of the virus which could be passed serially for fourteen generations on the rabbit. The disappearance of the filterability is therefore a property of the strain of virus. Furthermore, Friedewald and Kidd (1944), in attempts to pass the virus by methods perfected by one of them, found that many papillomas of the domestic rabbit could be passed. Thus, many such rabbits do actually contain the virus, but in very small amounts. This should not be taken as the normal finding, for Rous, Kidd, and Smith (1952) state that over half of their strains contain no infective virus, even with the method of Friedewald.

However this may be, the researches on the immunology of the Shope papilloma should be taken up again in the light of these later findings to determine whether or not the antigenic properties are indeed due to the minute amounts of virus that may be present. If so, the proofs advanced for the existence of a masked virus become valueless. This would seem to be unlikely, however, for as will be shown the immunological reactions are maintained for many generations in the grafts of the cancers that develop from these papillomas, although all trace of free virus seems to have gone.

To study the behavior of the virus in these cancers, Kidd, Beard, and Rous (1936) and Kidd and Rous (1940) grafted the epitheliomas that arise from them into other rabbits. One such strain, V2, has been maintained for 13 years over seventy-three passages (Smith, Kidd, and Rous,

1952). During five years, which comprised twenty generations, over one hundred young rabbits were successfully inoculated, and each developed a complement-fixing antibody to this antigen in its blood (Kidd, 1950). The same result was obtained even after multiple passages through animals hyperimmunized against the virus. This led Kidd (1942) to conclude that the Shope virus was masked in the cancers that derive from the papillomas and was not acting in the manner of an accidental contamination of the tumor ("inapparent passenger"), but that it was always in an "enduring partnership" with the cell. The passages were continued during the World War II, but lack of material prevented the continuation of the serological tests. When these were resumed, four and one-half years later, at the forty-sixth passage, antibodies were no longer present in the serum of the tumor-bearing animals, and they have not been detected since (Smith, Kidd, and Rous, 1948). The absence of virus, even in the masked form, was confirmed by Ginder and Friedewald (1952), who grafted the tumor V2 into the anterior chamber of the eye of cottontail rabbits and found that it grew in 75% of the animals without any antibody to Shope virus being detectable. The virus had either completely disappeared or had taken a further step in simplification by losing not only its particulate nature but also its antigenic structure. This latter receives some support from the most recent researches which indicate that the cells of the V2 tumor contain a protein that is antigenically different from the Shope virus but foreign to the host.

Smith, Kidd, and Rous (1952) took up this work again, mainly to see whether the perfected methods would enable them to detect free virus in the cancers arising from the papillomas. To give themselves the best chance they used tumors arising from papillomas in which free virus could be detected. To eliminate antibody action, the tumors were taken only after perfusion of the animals, and the grafts were made into newly born rabbits where the cancers develop so rapidly that enough material for the tests could be obtained before antibodies had time to appear. Of the four transplantable tumors thus obtained, V3–V6, only V3 produced minute papillomas in three tests, indicating the presence of very small amounts of virus; no trace was found in the other three. This appears to suggest that the formation of the tumor usually coincides with the disappearance of the virus in its transmissible form. A later epithelioma, V7, studied by Rogers, Kidd, and Rous (1950), reacts like the V2 in its earlier stage; that is, it contains virus in the masked form inducing antibodies in the host bearing it.

To investigate the behavior of the virus in tumors of the cottontail rabbit, Syverton, Wells, Koomen, Dascomb, and Berry (1950) inoculated the tumors arising in the wild rabbits into Eastern cottontails, in which

spontaneous Shope infections have never been seen. No tumor resulted, and the animals contained no virucidal antibodies. The absence of free virus from these tumors is the more striking since the papilloma which underwent the malignant change contained large amounts of virus.

From these facts it seems that two interpretations are possible. Syverton and his collaborators consider that in the cottontail the infection by Shope virus undergoes three successive stages: a proliferating stage coinciding with the development of the papillomas, a stationary phase indicating the culmination of the disease, and, finally, the disappearance of the virus which may coincide either with the regression of the papillomas or the malignant transformation. Thus Syverton and his collaborators conclude that even if some day the virus should be found in a cancer of the cottontail it should be regarded as an incidental passenger without any effect upon the cancers as such. From this point of view the condition in the domestic rabbit is an infection poor in virus, coinciding with marked proliferation which may frequently end in cancer and the disappearance of the virus. In this connection they sharply criticize the conclusions of Rous concerning the masked viruses in cancers, indicating that all this doctrine is based upon but two cancers serially transplanted.

To most people this attitude will seem illogical, for as it is agreed that the papilloma is due to the virus one thus arrives at the paradox that the greatest proliferation results when the virus is present in small amounts, and especially when it is not there at all. And this is in direct contradiction to the fact that it is the most virulent strains, the heaviest infections (Rous, Beard, and Kidd, 1936), and the most vigorous and rapidly growing papillomas that predispose for the malignant transformation. This interpretation might be justified if the existence of masked viruses were unknown. But since many examples are now known of virus existing in an incompletely antigenic form, and since also the Shope virus has an exceedingly narrow cytotropism, indicating highly exacting requirements for its environment, it seems more reasonable to hold the exactly opposite idea. One may consider that the domestic rabbit offers a particularly favorable site for the rapid proliferation of the cells, but it is precisely this rapid proliferation which prevents complete synthesis of the virus, as in the rapid papillomas, the cottontail cancer, and also the sarcomatous stage of the Shope fibroma. It may be that the rapidly growing cells lack something that is necessary for the complete synthesis of virus. Anyhow, virologists are aware of clear examples of such synthesis of incomplete virus, such as influenza when inoculated in large amounts into the egg. We thus return to the idea previously enunciated by Shope,

that the masked virus plays an important role in the infection by this virus.

2. Papillomas of the Mucosa of Various Animals

A buccal papillomatosis of the dog has long been known; in 1898 Penberthy proved its contagious nature, and experimental transmission has been obtained (Salomon-Balssa, 1939). These papillomas appear after an incubation period of 4 to 6 weeks and disappear after a stationary phase of about the same duration, leaving a permanent immunity. De Monbreun and Goodpasture (1932) made an extensive study of the causal agent, a virus which passes the Berkefeld N filter. Dried preparations remain active after 63 days in the cold and 84 days in 50% glycerol. The virus will withstand heating for 1 hour at 45° but loses its activity at 58°. Its exacting specificity is indicated by the fact that inoculation succeeds only upon the buccal mucosa, and attempts on other mucosa such as the vagina or conjunctiva have always failed.

A comparable specificity is shown by another oral papillomatosis found in domestic rabbits in the region of New York and studied by Parsons and Kidd (1936, 1943). This consists of small, discrete, benign papillomas occurring on the under surface of the tongue. It is only slightly contagious, although due to a virus which will pass Berkefeld V and N filters. The period of incubation is 6 to 38 days after inoculation, and regression follows after two to thirteen months, leaving the animal resistant to further inoculation.

The virus can be preserved for 40 days at −22° or 703 days in 50% glycerol in the cold. It withstands heating to 65° for 30 minutes, but not 75°. This virus will reproduce the papillomas on the oral mucosa of several species of rabbits and of the hare, but not on skin or other mucous membranes such as the nasal, conjunctival, or genital; thus it differs from the Shope papilloma which acts upon skin and not mucosa. There is no cross-immunity between the two.

In the natural state these papillomas are often found in rabbits whose mothers carried the lesion, as the virus can be passed from the mother to offspring during suckling. Furthermore it can remain in the mouth in a latent condition and appear only after injury to the mucosa, such as that induced by rough food or trauma. Tar seems to be an effective adjuvant, since spontaneous oral papillomas are much more frequent in tarred animals which lick their ears or feet. The papillomas never appear in tarred wild cottontail rabbits, although these animals are susceptible to the virus.

3. Cutaneous Papillomas of Various Animals

Cutaneous papillomas are found not only in rabbits but also in other species such as the horse (Cadeac, 1901), the cat (Creech, 1929), and the rat (Roussy, Guérin, and Guérin, 1945) and are comparable with human warts.

The papilloma of horses is an infectious growth normally restricted to the skin of the nostrils and lips. It is transmissible by extracts and filtrates through a Mandler filter and progresses for two months after an incubation period of two to three months. Some immunity is produced as a result of experimental infection, but a more solid one after natural infection. The causal agent will withstand 45° for half an hour, but is inactivated at 50°. It can be kept in 50% glycerol for 73 days at 4° but becomes inactive after 112 days. Kept at −35° it will remain active for 185 days but not for 224 days (Cook and Olson, 1951).

This agent seems to resemble the one inducing papillomas of the cat, which usually occur on the head and shoulders. In addition, this virus is capable of producing papillomas when transferred to cattle. If horses aged one to three years are inoculated with these, papillomas are induced which regress after growing for 30 to 50 days. In one horse a very persistent growth which endured for 600 days transformed into a sarcomatoid resembling the equine sarcoids described by Olson (1948). It recurred three times after removal but never produced metastases. Re-inoculated into another horse, it reproduced the lesion. Filtrates produced the condition in one horse, and glycerinated material was still active after two years (Olson and Cook, 1951).

It should be noted that there is no antigenic relation between the agents of the various papillomas of the rabbit, cat, cattle, dog, and man (Beard and Kidd, 1936).

4. Shope's Infectious Fibroma of Rabbits

This well-known infectious fibroma was first described by Shope (1932a,b) and studied in detail by Ahlström (1938). It is easily transmitted to the wild or domestic rabbit by subcutaneous or intratesticular grafts but is usually transmitted by Berkefeld filtrates.

The average size of the virus varies between 125 and 175 mμ (Schlesinger and Andrewes, 1937). Paschen (1936) demonstrated elementary bodies in fibroblasts of the infected rabbit, in tissue cultures, and on the allantoic membrane. Similarly, Ledingham (1937) found elementary bodies comparable with those of vaccinia by ultracentrifugation. According to Herzberg and Thelen (1938) and Herzberg and Wedemann (1940)

the particles are smaller than those of variola and can be found also in epithelial cells after inducing keratoconjunctivitis by inoculation upon the rabbit cornea. But it should be noted that Smith (1948) successfully passed the virus on the chorioallantoic membrane without ever finding any visible lesions or elementary bodies.

The virus can be preserved for long periods in glycerol, but it is rapidly destroyed by heating at 55°. The disease is not contagious and not transmitted by the male to the female or by the mother to her offspring. After recovery from the fibroma the animals have a solid resistance and their serum is strongly viricidal.

If the ordinary virus is injected into an animal previously treated with tar, even with just one dose, veritable neoplasms are produced (Ahlström and Andrewes, 1938a). After intravenous injection of the virus a generalized fibromatosis results which may be fatal. After subcutaneous injection a sarcoma results, in which the virus may be absent (Ahlström and Andrewes, 1938b). The same result is obtained with hydrocarbons such as benzpyrene or with x-rays (Clemmesen, 1938).

Another interesting fact is the relationship of this fibroma virus with the virus of infectious myxoma of Sanarelli, as shown by cross-immunity tests (Shope, 1932b, 1936a,b, 1938; Berry and Lighty, 1936). The protection which an inoculation with fibroma gives against myxoma, a disease always fatal, appears with astonishing rapidity. It becomes effective 2 or 3 days after inoculation (Hyde, 1939) and thus seems to be due to an interference phenomenon rather than to a true immunity. The relationship between the two viruses is seen again in the extremely interesting experiments of Berry and Dedrick (1936) concerning the reactivation of killed myxoma by living fibroma virus. These experiments, successfully repeated in several laboratories, can doubtless be explained by a recombination similar to that which may occur between two strains of bacteriophage. Margaret Smith (1952), in a detailed study of this phenomenon, concluded that heat destroys the myxoma virus, leaving only a fraction free from protein and resisting the action of alcohol, which was probably desoxynucleic acid. It could be this fraction which reacts with the fibroma virus to convert it into myxoma virus, or equally well it could be that it is the constituents of the fibroma virus which can complete the nucleic acid to make a myxoma virus.

Furthermore, Duran-Reynals (1940) showed that the fibroma virus could produce lesions in the rabbit whose relation with myxoma was particularly striking if newly born animals were used. If large doses were injected, a necrotic inflammation resulted which was rapidly fatal. With smaller doses the inflammatory process showed more and more a proliferative character, and a whole series of lesions were observed in which the

lesions were successively inflammatory, sarcomatous with metastases, generalized fibromatosis, and finally the classical form of Shope.

Besides these facts emphasizing the close relationship of the two viruses, there are others indicating the differences between them. Their antigenic constitution is not identical, for cross-immunity tests using the complement fixation technique indicates that each has a specific antigen (Andrewes and Ahlström, 1938). In general, the myxoma virus behaves both *in vivo* and *in vitro* as a better antigen, both quantitatively and qualitatively, than the fibroma virus. The latter is not transmitted by contact (Hyde and Gardner, 1939) like the myxoma, and its sensitivity to radiant heat is less (Thompson, 1938). A clear difference exists as far as virulence is concerned (Nauck, 1937); fibroma tissue rarely contains more than 3000 infectious doses per gram, whereas tissue often contains more than a million.

As Nauck suggests, it might therefore seem that the two viruses are but variants of a single virus which have resulted from mutation, a hypothesis which seems especially plausible, as both viruses have a tendency to form variants. By intracerebral passage of myxoma, Hurst (1937a) obtained a variant of the virus (neuromyxoma) which was no longer fatal when inoculated intradermally into rabbits. This property was stable and not reversed by intratesticular passage. The neuromyxoma induced a much more benign disease than the classical form, of which it showed all the characters except the formation of myxomatous cells. Hurst (1937b) failed to reproduce the results of Berry by inoculating a mixture of neuromyxoma and fibroma viruses.

On the other hand, during the experiments with a mixture of active fibroma and inactive myxoma, Berry (1938) obtained a strain 80 A Shope which had a low mortality (15%); this strain appeared suddenly and retained its characteristics for twenty-three passages in rabbits during thirteen months. A point of some importance concerning the theory of Berry was that this strain when inactivated by heat and mixed with fibroma produced in rabbits a myxoma virus with the characters of strain 80 A.

The virus of fibroma may also show mutations, as Andrewes and Shope indicated (1936). They obtained several strains, one of which produced purely inflammatory lesions and another lesions of mixed inflammatory and fibromatous type, as though it were a mixture of two viruses (Andrewes, 1935). When these two strains were passed in cottontail rabbits, the mixed type caused pure fibromatous lesions, while the other continued to cause the purely inflammatory reaction (Shope, 1935c).

Recently, Kilham, Herman, and Fisher (1953) found cutaneous fibromas in the grey squirrels of Maryland which could be transmitted to

other squirrels by intracutaneous injection, then for four passages on woodchucks, and finally for two passages on domestic rabbits. Serological tests indicated that there was a cross-immunity between the squirrel fibroma and that of Shope.

5. Milk Factor

When a strain of mice with a high incidence of mammary cancer is crossed with a strain of low incidence, the result should be the same independently of whether the male or female came from the high line. Actually, however, the maternal influence is always found to be predominant; i.e., the number of tumors is always very much greater when it is the mother who belongs to the cancer line. This result, first shown by Lathrop and Loeb in 1918, was afterwards confirmed by many geneticists, e.g., MacDowell and Richter (1935), Little (1936), and others. This was clearly the case in so many investigations that some workers, such as Little, completely rejected Mendelian inheritance for mammary cancer of mice and invoked the action of extrachromosomal factors. Korteweg (1936) suggested three possibilities for the transmission of such an influence: through the cytoplasm of the ovum, across the placenta, or in the milk. Korteweg was in favor of the first of these possibilities, without being able to prove it. Fekete and Little (1942) indicated some facts in favor of an intrauterine transmission by transferring eggs from a "cancerous" female to a "noncancerous" one, and vice versa. The results were not convincing because the young mice were suckled by the mother in whose uterus they had developed and not by the mothers of their line, so that milk influences could not be eliminated.

In 1936 Bittner showed that the extrachromosomal factor was transmitted by the mother's milk. By fostering mice of the "cancer" strains on "noncancer" mothers, the cancer incidence was reduced from 97.4% for C3H mice to 1.9% in ten generations. Andervont (1944) obtained the complete elimination of mammary cancer in C3H mice in five generations by removing the animals from the uterus by Caesarian section and fostering them upon C57 mice free from the cancer. Conversely, the frequency of cancer in the latter line was raised from 9 to 70% by fostering them upon mothers of the cancer line. Something very important is thus transmitted by the milk, and intensive work was undertaken to determine the properties, nature, and role of this factor in the production of cancer.

A. *Properties of the Milk Factor.* Veritable mouse dairies have been organized in several laboratories of the United States for mechanically milking mice to supply the needs of various researches (Graff, Moore, Stanley, Randall, and Haagensen, 1948). The milk factor will pass Seitz and Berkefeld N filters (Andervont and Bryan, 1944, 1945). Chromato-

graphic purification resulted in a product whose activity corresponded to 0.004 μg. of nitrogen per infective dose (Dmochowski and Stickland, 1950). Recently, Dmochowski and Passey (1952) undertook purification by electrophoresis and thus obtained a mammary tumor from the anodic fraction containing 10^8 particles.

The results obtained by ultracentrifugation have been extremely variable, since the starting material was often very different; some used extracts of lactating glands (Bryan, Kahler, Shimkin, and Andervont, 1942; Visscher, Green, and Bittner, 1942); others used extracts of mammary tumor (Kahler and Bryan, 1943; Barnum, Ball, and Bittner, 1946, 1947; Barnum and Huseby, 1950); and others used milk treated with chymotrypsin (Graff, Moore, Stanley, Randall, and Haagensen, 1949). It would seem that complete sedimentation requires a centrifugal force of 120,000g (Passey, Dmochowski, Astbury, Reed, and Johnson, 1950, 1951).

The results obtained with the electron microscope are also contradictory, which will surprise nobody acquainted with the difficulties and uncertainties of this method of identifying viruses. At the present moment, two divergent opinions are held by two different groups of workers. Graff, Moore, Stanley, Randall, and Haagensen (1948, 1949) identify the milk factor with spherical particles of size 100 mμ, and therefore much smaller than those observed by Porter and Thompson (1948, 1949). On the other hand, Passey, Dmochowski, Astbury, and Reed (1947) and these workers with Johnson (1950, 1951) found spherical particles of 20 to 120 mμ, usually not larger than 30 mμ. The 20 mμ particles were found only in very malignant strains, and the carcinogenic activity was always associated with the 24-mμ particles.

Attempts at purifying the milk factor indicated that it was not destroyed by pepsin, acetone, or petrol ether (Bittner, 1945). Antibiotics do not seem to influence its action, for neither penicillin (Bennison, 1949) nor aureomycin has any action, although streptomycin will decrease the age at which tumors appear in line C mice (Malingren and Law, 1951). Glycerol extracts of tumor kept at 8° will retain activity for a week, but the activity is lost after the third month (Andervont and Bryan, 1944–1945). It will withstand 37° for an hour and remains active in milk kept at 8° for a fortnight (Andervont and Bryan, 1944; Bittner, 1944a). If dried at ordinary temperatures it rapidly loses its activity and will no longer produce tumors after a week (Bittner, 1944a). Lyophilized material will remain active for six months (Bittner, 1944a) or a year (Dmochowski, 1946). It is destroyed by heat in 30 minutes at 61 to 66° in milk or 56 to 66° in tumor extract. It can be preserved at −79°, and frozen extracts

are as active as fresh (Bittner and Imagawa, 1950). In tumor extracts it is active between pH 5.5 and 10.2 but inactivated at 4.5.

Barnum and Huseby (1950) found no significant chemical differences between mammary glands with or without the factor. But by treating the microsomal fraction from glands containing the virus at different pH's, they divided it into two parts: one of nucleoprotein, almost devoid of activity, and a lipoprotein containing almost all the activity. This does not mean that the milk factor does not contain nucleic acid, but rather that 95% of the nucleic acid of the microsomal fraction could be removed without destroying the activity of the agent (Bittner, 1948c). Despite their activity at high dilution the preparations were still too impure for a chemical study of the active agent.

B. *Immunology.* Green, Moosey, and Bittner (1946) injected ultracentrifugates of mammary cancer into rabbits and rats and obtained antisera which neutralized the milk factor, whereas antisera prepared against normal mouse tissues had no such action. Imagawa, Green, and Halvorson (1948) injected mammary tumors into rabbits and obtained specific antibodies which neutralized, precipitated, and gave a complement fixation reaction with the Bittner agent. Antibodies to normal mouse mammary glands did not have the same properties and showed a distinct difference in their precipitating power, which was very weak compared with the high titers obtained with mammary tumor extracts. It can thus be concluded that the Bittner agent is antigenically different from the normal mammary tissue of mice, as Heidelberger, Graff, and Haagensen (1952) also concluded from their serological studies using highly purified milk factor. Guinea pigs will also produce antiserum when injected with mouse tissue containing milk factor (Imagawa, Bittner, and Syverton, 1950). On the other hand, the milk factor has only a very weak antigenic action in the mouse itself. Gorer and Law (1949) obtained neutralization of the agent with heated serum from strain A high-cancer mice, but not with unheated A serum or with unheated or heated serum from hyperimmunized C57 black mice. Dmochowski and Passey (1952) found the serum of mice to be always devoid of antibodies, in both high and low cancer lines.

C. *Culture on Eggs.* We shall not enter into the controversy concerning the work of Taylor and his collaborators (1942–1949). The most interesting fact that emerged was the demonstration by Bittner, Evans, and Green (1945) that the milk factor could survive 12 days in the fowl embryo independently of the presence or absence of mouse cells. The concentration in yolk sac may be greater than in the tissue of origin of the mouse.

Altogether, the results of these researches show that the factor has all the properties of a virus, and since this is almost unanimously accepted now, it would be convenient to call it by the name of its discoverer as the Bittner virus or "Bittner agent." This seems to us not only just but rational if confusion with other factors transmitted by the milk is to be avoided.

D. *Experimental Methods.* To detect the milk factor it is necessary to select mice which have all the requirements for the production of mammary cancer, except, of course, the Bittner virus itself. In fact, nothing shows the complexity of research in this field better than the difficulties encountered in finding such material. Newly born mice from a "cancer" line which have been fostered upon a mother of a "non-cancer" line are obviously suitable, if it is possible to separate the mice from their mother at the moment of birth and find a suitable foster mother. This being done, it was natural to consider using such mice to begin a line free from cancer. Theoretically, there is nothing against this, but in practice cancer appears sooner or later in these lines after several generations. Virus may have been present from the outset, or introduced later, in amounts too small to cause a tumor; but the favorable situation allows the virus to multiply and the tumors then appear. The use of lines of mice free from the cancer might be considered, but these lines are usually not susceptible to the virus and therefore cannot be used to detect it. Andervont (1945) suggested that a better test object was provided by hybrids from the first generation of a cross between males of a high cancer line such as C3H and females of a low line such as I. The females could be used as they were, and the males after receiving an implant of estrogenic hormone to stimulate the mammary glands. As we shall see later, these mice, and especially their descendants, also have some surprises ready.

The milk factor should be given in the first 15 days of life (Bittner, 1942, 1943; Dmochowski, 1945), since adults are much more resistant to the virus, although this resistance can sometimes be broken down by repeated injections or by a single massive dose injection of virus. The material to be tested for the presence of virus can be given by a dropping-tube by mouth or by nasal instillation (Begg, 1949). If it is sterile, it is better to employ subcutaneous or inteperitoneal injection, the latter being much more efficient (Andervont and Bryan, 1944). When milk is used, 0.1 ml. is usually enough to produce tumors in susceptible animals (Andervont, Shimkin, and Bryan, 1942).

The second great difficulty in all work upon the Bittner virus lies in the fact that it is necessary to wait ten or twenty months for the result, i.e., the age at which the treated mice will produce tumors. In the intervening time there is nothing to indicate the presence of the virus. The

treated animals are not ill, and they gain weight like the controls. Neither is there any effect upon the sexual activities such as puberty, estrous cycle, and menopause that is characteristic for each line (Armstrong, 1948). Naturally, workers have sought for earlier changes in the mammary gland that might indicate the presence of the Bittner virus, but many such attempts (Van Gulik and Korteweg, 1940; Shimkin and colleagues, 1941, 1943; Bonser, 1945; Smith, 1945; Huseby, Bittner 1945; Pullinger, 1947) have not resulted in finding any modification sufficiently constant to indicate the presence or absence of the virus. They showed that hyperplastic nodules were especially frequent in the mammary glands of mice bearing the milk factor. Recently, Pullinger (1952) showed that these nodules were most often found in the second pair of glands, considering that they were pathognomic of the virus only if they were found in females less than one year old (Pullinger, 1952). After unilateral blockage of the nipples, Fekete, Little, and Richardson (1952) also showed that these foci of hyperplasia appeared only in females bearing the milk factor, and that they appeared especially on the blocked side. From the results of an extensive study of tumors arising in the absence of the Bittner agent in the line DBAb, Mühlbock, van Ebbenhorst, Teugbergen, and van Ryssel (1952) concluded that the agent was solely concerned in the formation of the hyperplastic nodules and not in their malignant transformation.

E. *Distribution of the Virus.* The virus occurs in the milk at as high a concentration as in the mammary gland, which implies a rapid multiplication during lactation (Huseby, Barnum, and Bittner, 1950). It is present in the milk throughout lactation, and even in the nonlactating gland (Shimkin and Andervont, 1945). The concentration in the milk may vary in different lactations, for Bittner (1943, 1944b) found a difference in the tumor incidence in successive lactations. On the whole, the concentration increases with the number of pregnancies but decreases again in old females from the seventh or eighth litter. But these variations are not found in C3H mice (Andervont, 1944).

The virus does not occur only in the mammary gland. Although present in the milk, it is also distributed throughout the organism and has been shown to occur in the thymus, spleen (Bittner, 1939; Andervont, Shimkin, and Bryan, 1942; Prehn, 1952) liver, brain, (Bittner, 1948a) lungs, heart, and kidney (Dmochowski, 1949b). Woolley, Law, and Little showed in 1941 that it was present in the blood. It occurs only in small amounts in the serum and is concentrated in the blood cells (Bittner, 1944a). A hemolysin has been found in the red cells of susceptible mice, and also in extracts of their mammary glands, and certain facts suggest that the milk factor is concerned with this hemolysin (Adelsberger, 1951).

From the observations of Graff, Randall, Carpenter, and Haagensen (1946) it seems unlikely that whole blood will be a rich source of the milk factor, and they were unable to find it in the serum. The pathology of the milk factor is complicated by effects of genetic influences and hormones (Bittner, 1952). Actually, each strain of mice varies in its reaction to the Bittner virus, as to any other infectious agent, in offering more or less favorable conditions. Certain lines, such as the C57 black, may be infected and give tumors for several generations, but the conditions seem to be unfavorable for the virus and it is eventually eliminated. The hormonal influence is itself conditioned by hereditary factors that depend, according to Bittner (1952), on multiple genes. One of the most striking features is the favorable effect of parturition on the multiplication of the virus. The amount of virus usually increases at each pregnancy, and the method of "forced breeding" is the best way of demonstrating virus which is below the tumor-inducing level (Bittner, 1945; Mühlbock, 1950b). Heston and Deringer (1952a) even think that in certain conditions the breeding factor can substitute for the milk factor in the induction of mammary cancer.

An unexpected complication which may play a role as yet undetermined in the epidemiology of mammary cancer is the presence of the Bittner virus in the sperm. The observations of Andervont and Dunn (1948a,b) and Foulds (1949) suggested that the male may play a part in the transmission of the milk factor, and Bittner (1950) reached the same conclusion. Mühlbock (1950a) showed that the virus was present in sperm taken from the tail of the epididymis, so that the agent can be carried by the spermatozoa and transmitted to the female (Mühlbock, 1952b). Thus the hybrids derived from a noncancer mother and a cancer male are not perfect material for testing for the presence of the Bittner virus. They may be contaminated, as indicated by the observation of Andervont and Dunn (1948a) that 60% cancers may occur in such animals.

F. *Role of the Bittner Virus in the Production of Mammary Cancer.* The experiments with the Bittner virus show two separate and well-defined indications which clearly show the importance of the agent in the production of mammary tumors in mice. If all absorption of maternal milk is precluded, as Andervont (1944) did by removing the young mice by Caesarian section, then complete suppression of mammary cancer is obtained in the C3H line where the disease usually has an incidence of 97.4%. Conversely, by introducing the Bittner virus into the cancer-free line C, it is transformed into a high cancer line with an incidence of over 70% (Andervont, 1941). This last experiment also illustrates very well the multiplication of the agent, for its activity was as great in the first as in the thirteenth generation.

Inoculation of Bittner virus into a resistant line does not always induce cancer, but when it happens the animal remains contaminated and transmits the virus to its descendants. It may thus be found in resistant lines which have not shown a cancer for generations, a fact which should be remembered in connection with certain paradoxical findings.

The study of quantitative factors with the Bittner virus has given contradictory results. Andervont and McEleney (1939) showed that there was a direct relation between the amount of milk absorbed and the frequency and age of appearance of the induced tumors. Bittner (1952) injected mice from 22 to 126 days with extracts of mammary cancer and found no relation between the amount of agent injected and the number of tumors resulting. There seems, however, to be a level below which animals of a susceptible line will not respond, i.e., will not produce a tumor. This was shown by Andervont, who infected mice of a susceptible line with very small doses of the Bittner virus. None of them produced a tumor, but mammary cancers appeared in the descendants in the first or second generation. The virus thus seems to increase in successive generations, eventually attaining the level necessary for cancerization.

Another point not yet considered thus suggests itself. If the sperm may be contaminated, it is no longer possible to exclude transmission of virus by the egg, and this may explain certain paradoxical findings that have too readily suggested the formation of virus *de novo*. Mühlbock (1952a) insisted that the facts are not in favor of a direct transmission of the infection to the offspring by sperm, but usually by infection by the maternal milk.

The Bittner virus can be transmitted to all strains of mice, and it causes tumors in all, the percentage of tumors depending upon the concentration of the virus and the genetic susceptibility and hormonal state of the host. So far, mammary tumors have not been induced in wild mice of the *Peromyscus* genus, indicating a high degree of species specificity, but it occurs in wild mice and can be transmitted in them for four generations without inducing tumors (Andervont, 1952).

The milk factor occurs in spontaneous tumors which appear in low cancer strains. Bittner (1948b) found it in a spontaneous mammary tumor of the C57 line. On the other hand, Dmochowski (1951) failed to demonstrate it in spontaneous tumors of three lines, C57, Y, and P, but it was found in the descendants of cancerous C57 mice in which mammary tumors appeared with an increasing frequency (personal communication).

The results are not in agreement concerning the presence of the factor in grafts of mammary cancers. The observations of Andervont (1944) suggested that it persisted. Bittner (1944a, 1948b) similarly recovered it from tumor extracts at the tenth passage. It was not present in the 63 Ca

tumor at its four hundred and twenty-eighth passage (Dmochowski, 1948); it was found in a mammary tumor at the forty-second passage but not subsequently (Dmochowski, 1949a, 1952).

Hummel and Little (1949) found it intermittently during passage of an adenocarcinoma of line A, but it vanished at the thirty-fourth passage. It seems that the transplants grow more rapidly and the mice die sooner when agent is present (Barrett and Deringer, 1952; Barrett, Deringer, and Dunn, 1952).

Many investigations have been carried out to determine whether other mammary tumors, especially chemically induced ones, contain the Bittner virus (Kirschbaum and Bittner, 1945; Dmochowski and Orr, 1949; Andervont and Dunn, 1950). It would seem that the milk factor is not concerned with the induction of tumors such as those of the adrenal capsule (Woolley, Dickie, and Little, 1952) or with other cancerous conditions such as mouse leukemia (Miller and Pybus, 1945a,b). However, Rudali (1952) showed that mammary cancer could be induced in males of the R III line by a derivative of allenolic acid, if they had received milk factor from birth.

To conclude, it seems that the Bittner virus is now seen to be the agent of a well-recognized type of cancer: the spontaneous mammary tumor usually found in laboratory mice and so well described by the pioneers of cancer research such as Ehrlich, Apolant, Borrel, and others.

Bittner's discovery has a far-reaching importance for the problem of cancer viruses, especially because of the frequency of the condition which they cause. The domain of the virus-induced tumors suddenly extended in a spectacular fashion to include the most frequent spontaneous animal tumor and the one most often used for researches on cancer. The adversaries of the virus theory of cancer always sought to show that the tumors caused by viruses were of a special type and different from other neoplasms. But here we are concerned with an epithelial carcinoma of mammals which is in no way different from the others and, moreover, one for which the etiological problem seemed almost solved. The action of estrogens on a tissue hereditarily susceptible seemed to offer a satisfactory explanation without any need whatever for invoking a virus. But such a virus was discovered and shows all the characters that would be expected of a carcinogenic virus; that is, it is widely disseminated but its action is highly conditioned, and it has a very long latent period that may last not only for the existence of the individual but even over several successive generations. Thus the extreme complexity of the conditions upon which the production of the cancer depends makes it easy for us to understand the failures that always attended earlier experiments, where it was thought sufficient to inject a tumor filtrate to show the presence of a

virus. It was rather naïve to expect that a cancer virus should induce its tumor when and where it happened to be injected, yet it is upon the failures of such experiments that the validity of the virus theory of cancer is still being judged.

IV. General Discussion

To arrive at some general conclusion concerning the role of viruses in the causation of cancer, it is first necessary to know whether the agents responsible for the tumors that have been described in this review really are viruses. This might seem superfluous at first, but is necessary since certain authors have tried to make a class, separate from the other viruses, for the agents which induce the filterable tumors, calling them "viroids" (Altenburg, 1946) or "pro-virus" (Darlington and Mather, 1949). One thus runs immediately into the problem of defining what is meant by a virus. Such a definition is impossible at the present moment, but everyone will probably agree that the viruses can be considered as submicroscopic infectious particles which will multiply only inside living cells.

Nevertheless it must be admitted that researches in the last few years have shown two circumstances in which this definition breaks down for recognized viruses. The first relates to what is usually referred to as the masked condition, where the virus occurs not in a corpuscular form but dissociated and attached to cell structures, and it may even be asked whether the classic appearance of virus particles represents merely a phase in the developmental cycle, perhaps a resistant form as Anderson (1946) suggested, or more likely a form for dissemination. The second condition concerns the transmission of latent virus from one generation to another. This occurs with bacteriophage in lysogenic strains and with many plant and insect viruses such as with polyhedral disease, as Vago recently showed (1951). Here the exogenous infectious character of the malady ceases to exist, and the distinction between virus and cellular constituent becomes difficult, since it concerns a very wide-spread infection occurring in most of the species, strain, or race.

However, these are problems which in the last analysis concern not the tumor viruses but viruses in general, whereas we are concerned here with deciding whether the tumor viruses, considered as a group, have any properties by which they can be differentiated from other viruses. Claude and Murphy (1933), Haddow (1947), Kidd (1946a, 1950), Rhoads (1949), and others have claimed this, but most of the arguments they advanced have already been overthrown by facts and are no longer considered as valid even by those who advanced them.

Thus the objection that natural transmission of the agents, especially

in birds, was unknown and that there was no epidemiology of cancer viruses is refuted by the recent researches upon avain leucosis. And many other facts are also known in connection with this point. The importance of young animals as indicators of the blastogenic viruses, possible transmission by germ cells, as shown by Gross (1951) and called "vertical epidemics," the long latent period, the often fundamental role of genetical factors, all these supply elements for founding an epidemiology of cancer viruses much more comprehensive than that of shingles, herpes, and many other diseases of undoubted viral origin.

The objections based upon the antigenic properties of the blastogenic viruses are no better founded. It cannot be denied that, especially in the case of the bird tumors, the viruses are often associated with normal host constituents; but this is also the case with authentic viruses such as influenza, equine encephalitis, and other viruses. Undoubtedly the antigenic power of the Bittner virus in the susceptible animal—the mouse—is low. But even if totally lacking, it is not contrary to the idea of a viral agency, for it shares this character with other viruses such as the common cold.

It has been claimed that the narrow specificity of the agent, in the sense that it always reproduces the same tumor, is unlike the behavior of a real virus. But the same is found with true viruses. Nothing can be more constant than the appearance of molluscum contagiosum: yet such specificity is not found with all carcinogenic viruses, the Rous virus, for example, inducing very diverse lesions and even necrosis.

The morphogenic action of agents which enables them to induce from identical cells either myxomas, fibrosarcomas, or even osteosarcomas led certain authors to suggest that they were more like organizers than viruses. But the virus has done no more than evoke the latent potentialities of cells in the same way that inflammation can induce successively scar tissue, angiomatous formations, and even bone. Nothing can be more different, from the point of view of pure morphology, than a myxoma of Sanarelli and a tubercle, but both of these originate from the same cells by the action of undeniably infectious agents. There is therefore no need to invoke the hypothesis of organizers in this connection.

We may stop here and conclude that there is nothing so far as we know which enables us to differentiate the blastogenic agents from other viruses. There exists, therefore, a whole group of benign and malignant tumors and leukemias which are due to authentic viruses. But it is important to emphasize that this is not merely an addition of another etiological factor to those already known, such as parasites, radiations, and certain chemical carcinogens. Viruses occupy a special position because some of them, such as the Rous virus and certain leukemias, will cause the malig-

nant transformation in an infinitely shorter time than any other carcinogenic factor. They really are the direct agents of the cancerization, whereas the others produce this only after a more or less long delay.

This is one reason why it may be asked whether viruses may not be concerned in other neoplastic conditions where their presence has not yet been proved, which is equivalent to suggesting that the virus theory may supply an explanation for the causation of cancer. It is probably true, as Foulds (1940) has already said, that it is possible that we could know all about normal growth and the factors controlling it without having the least understanding of neoplastic growth. For here a new element is introduced, and many authors, such as Loeb (1937, 1944), Smith and Rous (1945), Kidd (1946a), Haddow (1947), and Butenandt (1952) consider that autonomous multiplication of particles or "duplicants" or the autocatalytic production of some chemical substance in the interior of the cell may be the cause of the new property of autonomous growth acquired by the neoplastic cell. But what they cannot accept is the concept that these particles of chemicals can be of exogenous origin. The repercussion of the work of Sonneborn (1943, 1947) on the "killer" factor is illuminating. Although it was thought that the endogenous nature of a transmissible factor was thus proved, even the most rabid adversaries of the virus theory of cancer enthusiastically accepted the possible homology of the agent causing malignancy and the "kappa" factor. For this reason it is interesting to see whether, at the present state of knowledge, the endogenous origin of autoreproducible particles comparable with viruses is conceivable.

For long enough the wide-spread idea that there was some analogy between the viruses and genes assigned speculations of this order almost exclusivity to the nucleus. But little by little the orientation altered, and under the influence of several discoveries described in a review by Haddow (1944) it is now chiefly in the cytoplasm that the origin of autonomous particles is sought. Of the many elements there, the chondriomes first attracted attention. Graffi (1940, 1941) thought that, under the influence of the carcinogenic hydrocarbons, the mitochondria, to which they attach themselves selectively, might undergo irreversible changes, marked by an ever-increasing degree of autonomy in their metabolic functions. This could continue until their functioning became completely emancipated and they would then be in all ways comparable with viruses. Starting from an entirely different point of view, the phytopathologists Woods and Du Buy (1941, 1943) reached the same conclusion. When studying the variegational diseases, they noted that the abnormal chloroplasts which are the cause of this condition could enter normal cells and replace the normal chloroplasts, thus propagating the disease. Since these plastids

are autoreproducible, these diseases therefore represent a model showing how derivatives of normal mitochondria could become infectious and behave like viruses. If this is accepted, then the demonstration of the ultrachondriome by Oberling, Bernhard, Fèbvre, and Harel (1951), similar to structures described by Porter and Thompson (1947), represents additional proof, for these are mitochondrial structures which show undoubted transitions to elements the size of viruses and usually showing evidence of division. Furthermore, the ultrachondriome is especially well developed in neoplastic cells.

Taken all together, these facts are certainly impressive, but there are serious objections to this way of looking at the problem. The sole experimental fact that Graffi had to support his theory was the fixation of the carcinogenic hydrocarbons by the chondrial elements, but since they are liposoluble this is not unexpected, and the fact that they are attached momentarily to any point does not mean that they act there. They also cause other disturbances, as well as with mitochondria, not least among the chromosomes (von Moellendorff, 1939); and in the chain of processes leading to cancer the irreparable damage to chromosomes is certainly more important than alterations to mitochondria, whose transitory nature is notorious (Nadson and Rochlin, 1933; Biebl, 1935; Zollinger, 1950). As far as the work of the plant pathologists is concerned, it should be remembered that the plastids belong only to the vegetable kingdom and that despite their autonomy and perennial nature, not shared by the chondriomal structures of animal cells, no relation between chloroplasts and plant cancers has even been suggested. The transfer of diseased plastids from one cell to another, described by Horning and Petrie (1927) and later by Du Buy and Woods (1943, 1945), was denied by Newcomer (1940) and O'Brien (1942). As for the variegations transmitted by grafting, Rhoades (1946, 1949) suggests that they themselves may be due to a virus. The transition between pathological plastids and infectious particles is not therefore proved. As for the ultrachondriome, those who described it did not consider that it corresponded with transitions between the chondriome and virus, but rather with the proliferation of a very young chondriome in cells reproducing rapidly.

For these reasons it seems that the transformation of mitochondrial elements into virus particles has no convincing arguments in its favor. In addition, since certain viruses such as Rous virus and avian leukemias are pathogenic for many species, it seems unlikely that mitochondrial derivatives could acquire the properties of the blastogenic viruses so far known.

Second, the cytoplasmic particles described by Claude (1943a,b) under the name of microsomes should be considered. From the beginning

their story was closely linked with the problem of endogenous viruses. Claude sought to determine whether or not there were particles in normal cells comparable with the infectious ones isolated by ultracentrifugation of Rous sarcomas. It soon became apparent that such particles existed and that they constituted a major part of the cytoplasm, characterized by a high content of lipids and especially of ribonucleoproteins, which makes them the cause of cytoplasmic basophily (Claude, 1946, 1947–1948; Brachet, 1952).

As soon as Porter, Claude, and Fullam (1945) developed the technique of examining cells in the electron microscope to a suitable state, they found vesicular granules in the cytoplasm of macrophages of normal fowls and in the Rous sarcoma which appeared to correspond with the elements isolated by ultracentrifugation. However, these structures, which caused the cytoplasmic basophily, were not only present in the form of granules, but in the cells of glands with very active protein synthesis (pancreas, salivary) they occurred in the form of filaments or sheets of ergastoplasm (Dalton, Kahler, Striebich, and Lloyd, 1950; Dalton, 1951; Bernhard, Gautier, and Oberling, 1951; Bernhard, Haguenau, Gautier, and Oberling, 1952) or in the form of tubes according to the recent study by Palade (1952). The earlier suggestion of Monne (1948) of an identity between microsomes and chromidia was thus confirmed.

If the possibility of an identity between these structures and virus is envisaged, the first question to be considered is whether these elements are capable of multiplication. The great quantitative variation noted for the basophilic components corresponding with cellular function render this probable (Bernhard, Gautier, and Oberling, 1951; Bernhard, Haguenau, Gautier, and Oberling, 1952; Gautier and Diomede-Fresa, 1953). The second important point is to know whether these particles are endowed with a genetical continuity. This is unknown, but certain facts suggest otherwise. The close dependence of the cytoplasmic basophily upon the activity of the nucleolus, as indicated by the work of Caspersson (1950) and Brachet (1952) and their schools, together with the recent results showing a banded structure of the nucleolus (Bernhard, Haguenau, and Oberling, 1952) are more suggestive of the nucleolus as being the main center of somatic inheritance.

The existence of "plasmagenes" (Darlington, 1939) which will maintain the distinctive characters of metazoan cells is so far in the realms of pure speculation, and this well indicates the standing of the plasmagene theory of cancer. Strong justly remarked (1949) that "a consideration at present dealing with a hypothetical relationship between a hypothetical plasmagene and cancer is consequently upon a very precarious footing." Need it be added that the only examples of plasmagenes in the animal

kingdom, quoted *ad nauseam* in all publications on the subject, are the kappa particles of Sonneborn (1943) and the genoids of L'Héritier (1948), which are a Rickettsia and a virus, respectively. Darlington (1948) was forearmed against this objection when he declared that there was nothing against a Rickettsia's being a plasmagene, since the only thing that differentiated a Rickettsia from a plasmagene was the mode of transmission. If this attitude is taken, then the concept of plasmagenes extends itself to organisms vastly more complex than viruses, but in doing so loses all significance in the present context.

If we leave aside speculations about plasmagenes and consider the basophilic structures of cytoplasm as we know them today, then it may be asked whether it is possible for them to be changed by some noxious agency, so that they would become autonomous and acquire properties which eventually would make it impossible to distinguish them from a virus. This is theoretically possible, but if it could be proved it would have such important consequences—for it would be nothing more or less than spontaneous generation of a virus—that before considering it seriously a modicum of proof should be required; and this has never been offered. It has often been thought to have been produced, but finally the error has been admitted.

Anyhow, if the carcinogenic agents can transform protoplasmic structures into elements resembling viruses, it is difficult to see why they do not produce agents with new and varied pathogenic actions. Yet in the many thousands of experiments where all types of cells have been treated with all sorts of carcinogens, nothing that was not already new has ever appeared. As far as viruses are concerned, they seem totally devoid of creative ability, and, far from producing new agents, seem rather to favor the action of those that already exist.

A final fact to consider is the extreme complexity of the transmissible agents, even those which seem the most simple. For example the transmissible leukemia of fowls, with its variable pathogenicity, cytotropism, and species specificity, must certainly have a very complex genetical organization. So if several dozen genetical units can be established for something as simple as a bacteriophage, then there must be more for the leukemia virus. To create this from some bits of degraded nucleoprotein seems to be the equivalent, on the submicroscopic scale, of the story of Van Helmont's mice.

Opponents of the virus theory may reply that abnormal production of protein need not go as far as the formation of particles having all the properties of true viruses, but that autonomous production of a protein may be enough to induce malignancy in the cell in which it occurs. This is essentially the hypothesis of Kidd (1946a). He had shown (1938, 1940)

that the Brown-Pearce tumor contained a protein of high molecular weight which gave a complement fixation reaction with the sera of rabbits previously injected with cells or extracts of the tumor. This reaction was specific and did not occur with rabbits injected with normal tissues, inflammatory tissues, or other rabbit tumors. This protein is readily deposited by ultracentrifugation, inactivated by heating to 65° for 30 minutes, and destroyed by trypsin and chymotrypsin. Inoculation of this into normal tissue, tarred tissue, or tissues infected by syphilis never produced a tumor. On the other hand, cells of the Brown-Pearce tumor treated with the antiserum grew either feebly or not at all when inoculated into rabbits. It is thus possible to vaccinate the rabbit against this tumor by treatment with this specific protein (Kidd, 1946a). A similar substance was found by Kidd (1946b) in the V2 carcinoma derived from a Shope papilloma after many passages. This new material was unrelated to the causal virus.

Kidd (1950) thus concluded that cancer may be the result of an autocatalytic production of such chemical substances which, by interfering with normal synthesis, induced anaplasia and malignant transformation of cells. This idea is certainly attractive, and it seems very likely that similar materials will be found in other cancerous cells. But it should be remembered that such substances conform to the definition of masked viruses. Moreover, the V2 indicates how a virus may become more and more intimately associated with the mechanisms of cellular synthesis and pass from the particulate condition to the level of a chemical compound. The converse process, i.e., the transformation of a cellular constituent into a masked virus, is a possibility that it is premature to deny categorically. It would be, in fact, endogenous formation of abnormal nucleoprotein. Much research remains to be done before we know all the ways in which nucleoproteins can become parasitic, which is probably the basis of carcinogenesis. But at the moment this is speculative, and it may even be asked whether it may not be precisely a foreign origin which endows the nucleoproteins with their independence, or in other words the autonomy which makes them the agents of cancer.

REFERENCES

Virus Tumors of Birds

Amies, C. R. 1937. *J. Pathol. Bacteriol.* **44,** 141–161.
Amies, C. R., and Carr, J. G. 1939. *J. Pathol. Bacteriol.* **49,** 497–513.
Amies, C. R., Carr, J. G., and Purdy, W. J. 1939. *Am. J. Cancer* **35,** 72–79
Andrewes, C. H. 1931. *J. Pathol. Bacteriol.* **34,** 91–107.
Andrewes, C. H. 1932. *J. Pathol. Bacteriol.* **35,** 407–413.
Andrewes, C. H. 1933a. *J. Pathol. Bacteriol.* **37,** 17–25.

Andrewes, C. H. 1933b. *J. Pathol. Bacteriol.* **37**, 27–44.
Andrewes, C. H. 1936. *J. Pathol. Bacteriol.* **43**, 23–33.
Andrewes, C. H. 1939. *J. Pathol. Bacteriol.* **48**, 225–227.
Bedson, S. P., and Bland, J. O. W. 1929. *Brit. J. Exptl. Pathol.* **10**, 67–70.
Begg, A. M., and Cramer, W. 1929. *Lancet* **217**, 697–698.
Bernhard, W., and Oberling, C. 1953. *Bull. assoc. franç. étude cancer* **40**, 178–185.
Borges, P. R. F., and Duran-Reynals, F. 1952. *Cancer Research* **12**, 55–58.
Borrel, A. 1903. *Ann. Inst. Pasteur* **17**, 81–122.
Borrel, A. 1926. *Compt. rend. soc. biol.* **94**, 500–502.
Bryan, W. R., Kahler, H., and Riley, V. T. 1945. *Am. Assoc. Advance. Sci. Conf. on Cancer*, pp. 40–53.
Bryan, W. R., Lorenz, E., and Moloney, J. B. 1950. *J. Natl. Cancer Inst.* **10**, 1215–1238.
Carr, J. G. 1946. *Brit. J. Exptl. Pathol.* **27**, 1–3.
Carr, J. G. 1947. *Proc. Roy. Soc. (Edinburgh)* **62**, 243–247.
Carr, J. G., and Harris, R. J. C. 1951a. *Brit. J. Cancer* **5**, 83–94.
Carr, J. G., and Harris, R. J. C. 1951b. *Brit. J. Cancer* **5**, 95–105.
Carrel, A. 1925. *Compt. rend. soc. biol.* **93**, 10–12.
Chinn, B. D. 1952. *Proc. Soc. Exptl. Biol. Med.* **80**, 359–360.
Claude, A. 1935. *J. Exptl. Med.* **61**, 41–57.
Claude, A. 1938. *Science* **87**, 467–468.
Claude, A., Porter, K. R., and Pickels, E. G. 1947. *Cancer Research* **7**, 421–430.
Des Ligneris, M. J. A. 1932. *Am. J. Cancer* **16**, 307–321.
Des Ligneris, M. J. A. 1935. *Compt. rend. soc. biol.* **120**, 777–780.
Doerr, R. 1937. *Z. Hyg. Infektionskrankh.* **119**, 635–659.
Doerr, R. 1949. Die Immunitätsforschung, 10th ed., Vol. 4, p. 145. Springer, Vienna.
Dmochowski, L. 1938. *Z. Immunitätsforsch.* **93**, 311–330.
Dmochowski, L. 1948a. *J. Natl. Cancer Inst.* **9**, 57–67.
Dmochowski, L. 1948b. *J. Natl. Cancer Inst.* **9**, 69–72.
Dmochowski, L., and Knox, R. 1939. *Brit. J. Exptl. Pathol.* **20**, 466–472.
Duran-Reynals, F. 1940a. *Yale J. Biol. Med.* **12**, 361–398.
Duran-Reynals, F. 1940b. *Yale J. Biol. Med.* **13**, 61–76.
Duran-Reynals, F. 1940c. *Yale J. Biol. Med.* **13**, 77–98.
Duran-Reynals, F. 1942. *Cancer Research* **2**, 343–369.
Duran-Reynals, F. 1943a. *Yale J. Biol. Med.* **16**, 53–57.
Duran-Reynals, F. 1943b. *Cancer Research* **3**, 569–577.
Duran-Reynals, F. 1946a. *Cancer Research* **6**, 529–534.
Duran-Reynals, F. 1946b. *Cancer Research* **6**, 545–552.
Duran-Reynals, F. 1947. *Cancer Research* **7**, 99–102.
Duran-Reynals, F. 1950. *Yale J. Biol. Med.* **22**, 555–564.
Duran-Reynals, F., and Estrada, E. 1940. *Proc. Soc. Exptl. Biol. Med.* **45**, 367–372.
Elford, W. J., and Andrewes, C. H. 1935. *Brit. J. Exptl. Pathol.* **16**, 61–66.
Engelbreth-Holm, J. 1942. Leukaemia in Animals. Oliver and Boyd, Edinburgh.
Epstein, M. A. 1951. *Brit. J. Cancer* **5**, 317–327.
Epstein, M. A. 1952. *Brit. J. Cancer* **6**, 254–261.
Feldman, W. H. 1932. Neoplasms of Domesticated Animals. W. B. Saunders, Philadelphia.
Felloni, G. 1930. *Pathologica* **22**, 256–259.
Foulds, L. 1934. *11th Sci. Rept. Imp. Cancer Research Fund Suppl.* pp. 1–32.
Foulds, L. 1937a. *J. Pathol. Bacteriol.* **44**, 1–18.
Foulds, L. 1937b. *Am. J. Cancer* **31**, 404–413.

Foulds, L., and Dmochowski, L. 1939. *Brit. J. Exptl. Pathol.* **20,** 458–465.
Fraenkel, E. 1930. *Z. Krebsforsch.* **32,** 167–170.
Fujinami, A., and Suzue, K. 1928. *Trans. Japan. Pathol. Soc.* **18,** 616–622.
Gereb, P., and Simke, R. 1932. *Z. Krebsforsch.* **35,** 73–77.
Greenwood, A. W., Blyth, J. S. S., and Carr, J. G. 1948. *Brit. J. Cancer* **2,** 135–143.
Gye, W. E. 1936. *Intern. Congr. Cancer (Bruxelles)* **1,** 48–56.
Gye, W. E., and Purdy, W. J. 1931. *The Cause of Cancer.* Cassell and Co., London.
Gye, W. E., and Purdy, W. J. 1933. *Brit. J. Exptl. Pathol.* **14,** 250–259.
Haddow, A. 1933. *J. Pathol. Bacteriol.* **37,** 149–155.
Hadfield, G., and Garrod, L. P. 1948. Recent Advances in Pathology, 5th ed., p. 114. Churchill, London.
Karnofsky, D. A., Parisette, C., Patterson, P. A., and Jacquez, L. 1949. *Acta Unio Intern. contra Cancrum* **6,** 641–651.
Kauffmann, F. 1929. *Z. Krebsforsch.* **30,** 136–138.
Lacassagne, A., and Nyka, W. 1938. *Compt. rend. soc. biol.* **128,** 1038–1040.
Laclau, M. C., and Pillado-Matheu, J. 1930. *Compt. rend. soc. biol.* **103,** 1285–1287.
Landsteiner, K., and Miller, C. P. 1924. *Proc. Soc. Exptl. Biol. Med.* **22,** 100–102.
Ledingham, J. C. G., and Gye, W. E. 1935. *Lancet* **I,** 376–377.
Lépine, P., and Lafforet-Furiet, J. 1943. *In* Les ultravirus des maladies animales, p. 989. C. Levaditi, P. Lépine, and J. Verge (eds.). Maloine, Paris.
Levine, M. 1939. *Am. J. Cancer* **36,** 276–302, 386–430, 581–602; **37,** 69–107.
Ludford, R. J. 1932. *10th Sci. Rept. Imp. Cancer Research Fund,* pp. 169–190.
Ludford, R. J. 1937. *Am. J. Cancer* **31,** 414–429.
McIntosh, J. 1933. *Brit. J. Exptl. Pathol.* **14,** 422–431.
McIntosh, J. 1935. *J. Pathol. Bacteriol.* **41,** 215–217.
McIntosh, J., and Selbie, F. R. 1939. *Brit. J. Exptl. Pathol.* **20,** 49–62.
Mauer, G. 1938. *Z. Krebsforsch.* **48,** 58–85.
Mellanby, E. 1934. *11th Ann. Rept. Brit. Emp. Cancer Campaign,* pp. 81–84.
Mellanby, E. 1938. *J. Pathol. Bacteriol.* **46,** 447–460.
Milford, J. J., and Duran-Reynals, F. 1943. *Cancer Research* **3,** 578–584.
Miszurski, B., Pikowski, M., Goldhaber, G., and Doljanski, L. 1945. *Cancer Research* **5,** 422–425.
Muller, J. H. 1931. *J. Immunol.* **20,** 17–23.
Murphy, J. B., and Claude, A. 1933. *Physiol. Rev.* **13,** 246–275.
Murphy, J. B., and Landsteiner, K. 1925. *J. Exptl. Med.* **41,** 807–816.
Murphy, J. B., and Rous, P. 1912. *J. Exptl. Med.* **15,** 119–132.
Murphy, J. B., and Sturm, E. 1941a. *Cancer Research* **1,** 477–483.
Murphy, J. B., and Sturm, E. 1941b. *Cancer Research* **1,** 609–613.
Murphy, J. B., Sturm, E., Favilli, G., Hoffman, D. C., and Claude, A. 1932. *J. Exptl. Med.* **56,** 117–129.
Nebenzahl, H. 1934. Étude expérimentale des tumeurs de la poule. Thèse, Librairie E. Le François, Paris.
Oberling, C., and Bernhard, W. 1952. *Verhandl. deut. pathol. Ges.* **35,** 89–96.
Oberling, C., Bernhard, W., Guérin, M., and Harel, J. 1950. *Bull. assoc. franç. étude cancer* **37,** 97–109.
Oberling, C., and Guérin, M. 1933. *Bull. assoc. franç. étude cancer* **22,** 326–359.
Oberling, C., and Guérin, M. 1943. *In* Les ultravirus des maladies animales, p. 1079. C. Levaditi, P. Lépine, and J. Verge (eds.). Maloine, Paris.
Oberling, C., and Guérin, M. 1950. *Bull. assoc. franç. étude cancer* **37,** 5–14.
Oker-Blom, N. 1951. *Acta Pathol. Microbiol. Scand.* **28,** 1–16.

Olson, C., Jr. 1940. *Mass. Agr. Expt. Sta. Bull.* **370,** 1–48.
Olson, C., Jr., and Bullis, K. L. 1942. Quoted by Duran-Reynals, F. 1946a.
Oshima, F., and Yabuuchi, Y. 1933. *Gann* **27,** 405–439 (Japanese).
Peacock, P. R. 1933. *J. Pathol. Bacteriol.* **36,** 141–152.
Peacock, P. R. 1935a. *Am. J. Cancer* **25,** 37–48.
Peacock, P. R. 1935b. *Am. J. Cancer* **25,** 49–56.
Peacock, P. R. 1936. *Leeuwenhoek Vereeniging (Amsterdam)* **III,** 91–94.
Peacock, P. R. 1946. *Cancer Research* **6,** 311–328.
Pentimalli, F. 1948. *Acta Unio Intern. contra Cancrum* **6,** 210–229.
Pikowski, M., and Doljanski, L. 1946. *Proc. Soc. Exptl. Biol. Med.* **61,** 246–248.
Purdy, W. J. 1933. *Brit. J. Exptl. Pathol.* **14,** 9–17, 260–267.
Riley, V. T. 1948. *Science* **107,** 573–575.
Riley, V. T. 1950. *J. Natl. Cancer Inst.* **11,** 199–214, 215–227, 229–235.
Ringsted, J. 1952. *Acta Pathol. Microbiol. Scand.* **31,** 275–280.
Rous, P. 1910. *J. Exptl. Med.* **12,** 696–705.
Rous, P. 1911. *J. Exptl. Med.* **13,** 397–411.
Rous, P., McMaster, P.D., and Hudack, S. S. 1935. *J. Exptl. Med.* **61,** 657–688.
Rous, P., Murphy, J. B., and Tytler, W. H. 1912a. *J. Am. Med. Assoc.* **58,** 1751.
Rous, P., Murphy, J. B., and Tytler, W. H. 1912b. *J. Am. Med. Assoc.* **59,** 1793–1794.
Rous, P., Robertson, O. H., and Oliver, J. 1919. *J. Exptl. Med.* **29,** 305–320.
Sandford, K. K., Likely, G. D., Bryan, R. W., and Earle, W. R. 1952. *J. Natl. Cancer Inst.* **12,** 1317–1344.
Sanfelice, F. 1927. *Zentr. Bakteriol. Parasitenk.* **103,** No. **1,** 415–419.
Shrigley, E. W., Greene, H. S. N., and Duran-Reynals, F. 1945. *Cancer Research* **5,** 356–364.
Sturm, E., and Murphy, J. B. 1928. *J. Exptl. Med.* **47,** 493–502.
Tenenbaum, E. 1943. *Brit. J. Exptl. Pathol.* **24,** 56–58.
Tenenbaum, E., and Doljanski, L. 1943. *Cancer Research* **3,** 585–603.
Teutschlaender, O. 1928. *Z. Krebsforsch.* **27,** 241–252.
Thomsen, O. 1939. *In* Handbuch der Virusforschung, Vol. II, pp. 994–1105. R. Doerr and C. Hallauer (eds.). Springer, Vienna.
Todd, C. 1930. *Proc. Roy. Soc. (London)* **B107,** 197–198.
White, A. W. M. 1927. *J. Cancer Research* **11,** 111–125.
Witebsky, E. 1929. *Z. Immunitätsforsch.* **62,** 35–73.
Yaoi, H., and Nakahara, W. 1935. *Japan. J. Exptl. Med.* **13,** 757–765.
Yoshikawa, H., and Ishimoto, N. 1930. *Trans. Japan. Pathol. Soc.* **20,** 701–703.

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Andersen, C. W., and Bang, O. 1928. Festskrift Bernhard Bang. Copenhagen.
Atanasiu, P. 1952. *Le Sang* **23,** 323–328.
Atanasiu, P., Vieuchange, M., and Strunge, B. 1951. *Acta Unio Intern. contra Cancrum* **7,** 213–215.
Bayon, H. P. 1931. Quoted by G. Lesbouyries, 1941. La pathologie des oiseaux-Vigot, Paris.
Beard, D., Eckert, E. A., Csaky, T. Z., Sharp, D. G., and Beard, J. W. 1950. *Proc. Soc. Exptl. Biol. Med.* **75,** 533–536.
Brewer, N. R., and Brownstein, F. 1946. *Am. J. Vet. Research* **7,** 123–128.
Burmester, B. R. 1945. *Poultry Sci.* **24,** 469–472.
Burmester, B. R. 1952. *Ann. N.Y. Acad. Sci.* **54,** 992–1003.
Burmester, B. R., and Cottral, G. E. 1947. *Cancer Research* **7,** 669–675.

Burmester, B. R., and Denington, E. M. 1947. *Cancer Research* **7**, 769–785.
Burmester, B. R., and Prickett, C. O. 1944. *Cancer Research* **4**, 364–366.
Burmester, B. R., Prickett, C. O., and Belding, T. C. 1946. *Cancer Research* **6**,189–196.
Cottral, G. E. 1951. *Proc. Natl. Acad. Sci. U.S.* (In press.)
Darcel, C. Le Q. 1952. *Brit. J. Cancer* **6**, 425–430.
Davis, O. S., Andrews, F. N., and Doyle, L. P. 1950. *Am. J. Vet. Research* **11**, 1428–1436.
Davis, O. S., Doyle, L. P., Walkey, F. L., and Cenker, L. K. 1947. *Poultry Sci.* **26**, 499–507.
Doljanski, L., and Pikowski, M. 1942. *Cancer Research* **2**, 626–631.
Eckert, E. A., Beard, D., and Beard, J. W. 1951. *Cancer Research* **11**, 245 (abstr.).
Eckert, E. A., Sharp, D. G., Beard, D., and Beard, J. W. 1952. *J. Natl. Cancer Inst.* **13**, 533–540.
Ellermann, V. 1921. Leucosis of Fowls and Leucemia Problems. Gyldendal, London.
Ellermann, V., and Bang, O. 1908. *Zentr. Bakteriol. Parasitenk.* **I, Orig. 46**, 595–609.
Engelbreth-Holm, J. 1935. *Klin. Wochschr.* **14**, 1677–1678.
Engelbreth-Holm, J., and Frederiksen, O. 1938. *Acta Pathol. Microbiol. Scand. Suppl.* **37**, 138–144.
Engelbreth-Holm, J., and Rothe-Meyer, A. 1932a. *Acta Pathol. Microbiol. Scand.* **9**, 293–332.
Engelbreth-Holm, J., and Rothe-Meyer, A. 1932b. *Z. Immunitätsforsch.* **74**, 347–352.
Engelbreth-Holm, J., Rothe-Meyer, A., and Uhl, E. 1935. *Acta Pathol. Microbiol. Scand.* **12**, 491–510.
Farkas, L. 1930. Quoted by Jármai, K. 1934.
Forestier, A. 1933. La leucémie transmissible des poules. These vétérin., Vigot., Paris.
Fox, H. 1923. Diseases in Captive and Wild Mammals and Birds. Lippincott, Philadelphia, London, Chicago.
Furth, J. 1932. *J. Exptl. Med.* **55**, 465–478, 495–504.
Furth, J. 1935. *J. Exptl. Med.* **61**, 593–615; **63**, 127–143.
Furth, J., and Breedis, C. 1935. *Arch. Pathol.* **20**, 379–428.
Furth, J., and Breedis, C. 1937. *Arch. Pathol.* **24**, 281–302.
Furth, J. and Miller, H. K. 1932. *J. Exptl. Med.* **55**, 479–493.
Gibbs, C. S. 1934. *Mass. Agr. Expt. Sta. Bull.* 308.
Greppin, O. G. 1937. *Bull. assoc. franç. étude cancer* **26**, 232–258.
Gross, L. 1951. *Cancer* **4**, 626–633.
Guérin, M. 1948. *Bull. assoc. franç. étude cancer* **35**, 423–426.
Guérin, M., and Guérin, P. 1947. *Bull. assoc. franç. étude cancer* **34**, 218–222.
Guérin, M., and Guérin, P. 1949. *Bull. assoc. franç. étude cancer* **36**, 199–209.
Haupt, H. 1928. *Berlin. tierärztl. Wochschr.* **34**, 158–159.
Hutt, F. B., and Cole, R. K. 1947. *Science* **106**, 379–384.
Jármai, K. 1930. *Arch. wiss. u. prakt. Tierheilk.* **62**, 113–131.
Jármai, K. 1932. *Arch. wiss. u. prakt. Tierheilk.* **65**, 46–71.
Jármai, K. 1933. *Deut. tierärztl. Wochschr.* **41**, 418–420.
Jármai, K. 1934. *Ergeb. allg. Pathol. u. pathol. Anat.* **38**, 227–312.
Jármai, K. 1935a. *Arch. wiss. u. prakt. Tierheilk.* **69**, 275–286.
Jármai, K. 1935b. *Arch. wiss. u. prakt. Tierheilk.* **70**, 62–70.
Jármai, K. 1938. *Arch. wiss. u. prakt. Tierheilk.* **73**, 295–300.
Jármai, K., Stenszky, T., and Farkas, L. 1932. *Arch. wiss. u. prakt. Tierheilk.* **65**, 46–71.
Jungherr, E. 1937. *Conn. Agr. Expt. Sta. Bull.* **218**, 1–47.

Kabat, E., and Furth, J. 1940. *J. Exptl. Med.* **71,** 55–70.
Kamarow, A. 1948. Quoted by Cottral, G. E. 1951.
Kirschbaum, A., Stern, K. G., and Hooker, C. W. 1940. *Yale J. Biol. Med.* **13,** 1–14.
Kögler, D. 1933. Quoted by Jármai, K. 1934.
Lhermitte, J., de Ajuriaguerra, and Souquet, 1943. *Bull. acad. méd. (Paris)* **127,** 632–637.
Lucas, A. M. 1946. *Am. J. Anat.* **79,** 431–471.
Lucas, A. M. 1949. *Am. J. Pathol.* **25,** 1197–1213.
Lucas, A. M., and Breitmayer, J. B. 1950. *Poultry Sci.* **29,** 450–461.
Lucas, A. M., and Oakberg, E. F. 1950. *Am. J. Pathol.* **26,** 75–111.
Lund, L. 1927. *Deut. tierärztl. Wochschr.* **35,** 51.
Oberling, C. 1937. *Bull. soc. franç. dermatol. syphilig.* **44,** 1193–1206.
Oberling, C., and Guérin, M. 1933. *Bull. assoc. franç. étude cancer* **22,** 180–212, 326–359.
Oberling, C., and Guérin, M. 1934a. *Bull. assoc. franç. étude cancer* **23,** 38–81.
Oberling, C., and Guérin, M. 1934b. *Le Sang* **8,** 892–924.
Oberling, C., and Guérin, M. 1943. *In* Les ultravirus des maladies animales, pp. 1079–1096. C. Levaditi, P. Lépine, and J. Verge (eds.). Maloine, Paris.
Olson, C., Jr. 1941. *Cancer Research* **1,** 384–392.
Olson, C., Jr. 1945a. *Am. J. Vet. Research* **6,** 103–106.
Olson, C., Jr. 1945b. *Cornell Vet.* **35,** 221–230.
Olson, C., Jr. 1945c. *Cornell Vet.* **35,** 308–313.
Olson, C., Jr. 1946. *Cornell Vet.* **36,** 41–47.
Olson, C., Jr. 1948. *Am. J. Vet. Research* **9,** 198–200.
Pappenheimer, A. W., Dunn, L. C., and Cone, V. 1929. *J. Exptl. Med.* **49,** 63–86.
Pappenheimer, A. W., Dunn, L. C., Cone, V., and Seidlin, S. M. 1929. *J. Exptl. Med.* **49,** 87–102.
Pentimalli, F. 1941. *Cancer Research* **1,** 69–70.
Pierce, M. 1942. *Am. J. Pathol.* **18,** 1127–1139.
Pikowski, M., and Doljanski, L. 1950. *Cancer Research* **10,** 1–7.
Pikowski, M., Goldhaber, G., and Doljanski, L. 1947. *Cancer Research* **7,** 393–397.
Potel, K. 1938. *Z. Infektionskrankh. parasit. Krankh. u. Hyg. Haustiere* **54,** 143–154.
Reinhardt, R. 1930. *Ergeb. Allg. Pathol. u. pathol. Anat.* **23,** 553–554.
Rothe-Meyer, A., and Engelbreth-Holm, J. 1933. *Acta Pathol. Microbiol. Scand.* **10,** 380–427.
Ruffilli, D. 1938a. *Boll. lega. ital. lotta contra tumori* **12,** 190–191.
Ruffilli, D. 1938b. *Boll. soc. ital. biol. sper.* **13,** 146–148.
Satterlee, G. R. 1906. *Proc. N.Y. Pathol. Soc.* **6,** 122–126.
Schoenaers, F. 1946. *Compt. rend. soc. biol.* **140,** 1235–1237.
Sharp, D. G., Eckert, E. A., Beard, D., and Beard, J. W. 1952. *J. Bacteriol.* **63,** 151–161.
Sharp, D. G., Eckert, E. A., Burmester, B. R., and Beard, J. W. 1952. *Proc. Soc. Exptl. Biol. Med.* **79,** 204–208.
Storti, E., and Zaietta, A. 1938. *Arch. sci. méd.* **65,** 897–940.
Troisier, J. 1934. *Bull. assoc. franç. étude cancer* **23,** 225–270.
Uhl, E., Engelbreth-Holm, J., and Rothe-Meyer, A. 1936. *Acta Pathol. Microbiol. Scand.* **13,** 434–452.
van den Berghe, L., and d'Ursel, P. 1939. *Compt. rend. soc. biol.* **131,** 1301–1302; 1302–1304.
Verne, J., Oberling, C., and Guérin, M. 1936. *Compt. rend. soc. biol.* **121,** 403–405.
Vigier, P. 1951. Thèse de méd. Techniques de culture dans l'oeuf embryonné et leur application à l'étude du cancer. Paris.

Vigier, P., and Guérin, M. 1951. *Bull. assoc. franç. étude cancer* **38**, 72–84.
Wakamatsu, Y. 1934. *Z. Infektionskrankh. parasit. Krankh. u. Hyg. Haustiere* **46**, 161–176.
Warren, D. C. 1951. Quoted by Cottral, G. E. 1951.
Waters, N. F. 1945. *Poultry Sci.* **24**, 226–233, 259–262.
Waters, N. F. 1947. *Poultry Sci.* **26**, 639–647.
Waters, N. F. 1951. *Poultry Sci.* **30**, 531–545.
Waters, N. F., and Bywaters, J. H. 1949. *Poultry Sci.* **28**, 254–261.
Waters, N. F., and Prickett, C. O. 1946. *Poultry Sci.* **25**, 501–508.
Winton, B. 1948. *Offic. Rept. 8th World's Poultry Congr.* pp. 712–718.

Tumors of Cold-Blooded Animals

Briggs, R. 1942. *Cancer Research* **2**, 309–323.
Briggs, R., and Grant, R. 1943. *Cancer Research* **3**, 613–620.
Champy, Ch., and Champy (Mlle.). 1935. *Bull. assoc. franç. étude cancer* **24**, 206–220.
Lucké, B. 1934a. *Am. J. Cancer* **20**, 352–379.
Lucké, B. 1934b. *Am. J. Cancer* **22**, 326–334.
Lucké, B. 1938a. *Am. J. Cancer* **34**, 15–30.
Lucké, B. 1938b. *J. Exptl. Med.* **68**, 457–468.
Lucké, B. 1952. *Ann. N.Y. Acad. Sci.* **54**, 1093–1109.
Lucké, B., and Schlumberger, H. G. 1940a. *J. Exptl. Med.* **72**, 311–320.
Lucké, B., and Schlumberger, H. G. 1940b. *J. Exptl. Med.* **72**, 321–330.
Lucké, B., and Schlumberger, H. G. 1949. *J. Exptl. Med.* **89**, 269–277.
Lucké, B., and Schlumberger, H. G. 1950a. *Cancer Research* **10**, 231–240.
Lucké, B., and Schlumberger, H. G. 1950b. *J. Natl. Cancer Inst.* **11**, 511–544.
Nigrelli, R. F. 1952. *Ann. N.Y. Acad. Sci.* **54**, 1076–1092.
Rose, M. S. 1952. *Ann. N.Y. Acad. Sci.* **54**, 1110–1119.
Rose, M. S., and Rose, F. C. 1952. *Cancer Research* **12**, 1–12.
Schlumberger, H. G., and Lucké, B. 1949. *Cancer Research* **9**, 52–60.
Weissenberg, R. 1949. *Cancer Research* **9**, 537–542.
Weissenberg, R. 1951. *Cancer Research* **11**, 608–613.

Shope Papilloma

Beard, J. W., Bryan, W. R., and Wyckoff, R. W. G. 1939. *J. Infectious Diseases* **65** 43–52.
Beard, J. W., and Rous, P. 1934. *J. Exptl. Med.* **60**, 723–740.
Beard, J. W., and Wyckoff, R. W. G. 1937. *Science* **85**, 201–202.
Fischer, G., and Syverton, J. T. 1951. *Cancer Research* **11**, 737–740.
Friedewald, W. F. 1942. *J. Exptl. Med.* **75**, 197–220.
Friedewald, W. F. 1944. *J. Exptl. Med.* **80**, 65–76.
Friedewald, W. F., and Anderson, R. S. 1940. *Proc. Soc. Exptl. Biol. Med.* **45**, 713–715
Friedewald, W. F., and Kidd, J. G. 1944. *J. Exptl. Med.* **79**, 591–605.
Ginder, D. R., and Friedewald, W. F. 1952. *Cancer Research* **12**, 429–432.
Greene, H. S. N. 1953. *Cancer Research*, **13**, 58–63.
Kahler, H., and Lloyd, B. J., Jr. 1952. *J. Natl. Cancer Inst.* **12**, 1167–1176.
Kidd, J. G. 1937. *Proc. Soc. Exptl. Biol. Med.* **35**, 612–614.
Kidd, J. G. 1938a. *Proc. Soc. Exptl. Biol. Med.* **37**, 657–658.
Kidd, J. G. 1938b. *Proc. Soc. Exptl. Biol. Med.* **38**, 292–295.
Kidd, J. G. 1938c. *J. Exptl. Med.* **68**, 733–759.
Kidd, J. G. 1939. *J. Exptl. Med.* **70**, 583–603.

Kidd, J. G. 1940. *Proc. Soc. Exptl. Biol. Med.* **43,** 770–772.
Kidd, J. G. 1942. *J. Exptl. Med.* **75,** 7–20.
Kidd, J. G. 1950. The Pathogenesis and Pathology of Virus Diseases. Columbia University Press, New York.
Kidd, J. G., Beard, J. W., and Rous, P. 1936. *J. Exptl. Med.* **64,** 63–67.
Kidd, J. G., and Parsons, R. J. 1936. *Proc. Soc. Exptl. Biol. Med.* **35,** 438–441.
Kidd, J. G., and Rous, P. 1938. *J. Exptl. Med.* **68,** 529–662.
Kidd, J. G., and Rous, P. 1940. *J. Exptl. Med.* **71,** 813–838.
Knight, C. A. 1950. *Proc. Soc. Exptl. Biol. Med.* **75,** 843–846.
Lacassagne, A. 1936. *Compt. rend. soc. biol.* **123,** 736–737.
Lacassagne, A. 1937. *Compt. rend. soc. biol.* **125,** 96–98.
Lacassagne, A., and Nyka, W. 1937. *Compt. rend. soc. biol.* **121,** 822–824.
Lauffer, W. A., and Stanley, W. M. 1938. *J. Biol. Chem.* **123,** 507–525.
Neurath, H., Cooper, G. R., Sharp, D. G., Taylor, A. R., Beard, D., and Beard, J. W. 1941. *J. Biol. Chem.* **140,** 293.
Rondoni, P., and Eisen, M. J. 1937. *Tumori* **23,** 511–532.
Rogers, S., Kidd, J. G., and Rous, P. 1950. *Cancer Research,* **40,** 237.
Rogers, S., and Rous, P. 1951. *J. Exptl. Med.* **93,** 459–488.
Rous, P., and Beard, J. W. 1934. *J. Exptl. Med.* **60,** 701–722, 741–766.
Rous, P., and Beard, J. W. 1935. *J. Exptl. Med.* **62,** 523–548.
Rous, P., Beard, J. W., and Kidd, J. G. 1936. *J. Exptl. Med.* **64,** 401–424.
Rous, P., and Kidd, J. G. 1938. *J. Exptl. Med.* **67,** 399–428.
Rous, P., Kidd, J. G., and Beard, J. W. 1936. *J. Exptl. Med.* **64,** 385–400.
Rous, P., Kidd, J. G., and Smith, W. E. 1952. *J. Exptl. Med.* **96,** 159–174.
Schlesinger, M., and Andrewes, C. H. 1937. *J. Hyg.* **37,** 521–526.
Selbie, F. R., and Robinson, R. H. M. 1948. *Brit. J. Cancer* **2,** 48–58.
Selbie, F. R., Robinson, R. H. M., and Shope, R. E. 1948. *Brit. J. Cancer* **2,** 375–379.
Sharp, D. G., Taylor, A. R., and Beard, J. W. 1942. *Proc. Soc. Exptl. Biol. Med.* **50,** 205–207.
Sharp, D. G., Taylor, A. R., Hook, A. E., and Beard, J. W. 1946. *Proc. Soc. Exptl. Biol. Med.* **61,** 259–265.
Shope, R. E. 1933. *J. Exptl. Med.* **58,** 607–624.
Shope, R. E. 1935. *Proc. Soc. Exptl. Biol. Med.* **32,** 830–832.
Smith, W. E., Kidd, J. G., and Rous, P. 1948. *Acta Unio Intern. contra Cancrum* **6,** 231 (abstr.).
Smith, W. E., Kidd, J. G., and Rous, P. 1952. *J. Exptl. Med.* **95,** 299–318.
Syverton, J. T., and Berry, G. P. 1935. *Proc. Soc. Exptl. Biol. Med.* **33,** 399–400.
Syverton, J. T., Dascomb, H. E., Koomen, J., Jr., Wells, E. B., and Berry, G. P. 1950. *Cancer Research* **10,** 379–384.
Syverton, J. T., Harvey, R. A., Berry, G. P., and Warren, S. L. 1941. *J. Exptl. Med.* **73,** 243–248.
Syverton, J. T., Wells, E. B., Koomen, J., Jr., Dascomb, H. E., and Berry, G. P. 1950. *Cancer Research* **10,** 474–82.

Papillomas of the Mucosa and Skin of Various Animals

Beard, J. W., and Kidd, J. G. 1936. *Proc. Soc. Exptl. Biol. Med.* **34,** 451–453.
Cadeac. 1901. *Bull. Sci. Vet. (Lyon)* **4,** 280–286. Quoted by Olson, C. 1948.
Cook, R. H., and Olson, C., Jr. 1951. *Am. J. Pathol.* **27,** 1087–1097.
Creech, C. T. 1929. *J. Agr. Research* **39.** 723–737. Quoted by Cook, R. H., and Olson, C., Jr. 1951.

De Monbreun, W. A., and Goodpasture, F. W. 1932. *Am. J. Pathol.* **8**, 43–55.
Olson, C., Jr. 1948. *Am. J. Vet. Research* **9**, 333–341.
Olson, C., Jr., and Cook, R. H. 1951. *Proc. Soc. Exptl. Biol. Med.* **77**, 281–284.
Parsons, R. J., and Kidd, J. G. 1936. *Proc. Soc. Exptl. Biol. Med.* **35**, 441–443.
Parsons, R. J., and Kidd, J. G. 1943. *J. Exptl. Med.* **77**, 233–250.
Penberthy. 1898. *J. Comp. Pathol. Therap.* **2**, 363. Quoted by Salomon-Balssa, L. 1939.
Roussy, G., Guérin, M., and Guérin, P. 1945. *Bull. acad. méd. (Paris)* **129**, 598–603.
Salomon-Balssa, L. 1939. La théorie infectieuse du cancer et la pathologie comparée des papillomatoses. Thèse vétérin. d'Alfort (Paris). Habauzit, Aubenas (Ardèche).

Shope's Infectious Fibroma

Ahlström, C. G. 1938. *J. Pathol. Bacteriol.* **46**, 460–472.
Ahlström, C. G., and Andrewes, C. H. 1938a. *J. Pathol. Bacteriol.* **47**, 65–86.
Ahlström, C. G., and Andrewes, C. H. 1938b. *J. Pathol. Bacteriol.* **47**, 87–99.
Andrewes, C. H. 1936. *J. Exptl. Med.* **63**, 157–172.
Andrewes, C. H., and Ahlström, C. G. 1938. *J. Pathol. Bacteriol.* **47**, 87–99.
Andrewes, C. H., and Shope, R. E. 1936. *J. Exptl. Med.* **63**, 179–184.
Berry, G. P. 1938. *J. Bacteriol.* **36**, 285–286.
Berry, G. P., and Dedrick, H. M. 1936. *J. Bacteriol.* **31**, 50–51.
Berry, G. P., and Lighty, J. A., Jr., 1936. *J. Bacteriol.* **31**, 49–50.
Clemmesen, J. 1938. *Acta Pathol. Microbiol. Scand. Suppl.* **38**, 47–48.
Duran-Reynals, F. 1940. *Yale J. Biol. Med.* **13**, 99–110.
Herzberg, K., and Thelen, A. 1938. *Virchow's Arch. pathol. Anat. u. Physiol.* **303**, 81–89.
Herzberg, K., and Wedemann, E. 1940. *Zentr. Bakteriol. Parasitentk.* **146**, 104–112.
Hurst, E. W. 1937a. *Brit. J. Exptl. Pathol.* **18**, 15–22.
Hurst, E. W. 1937b. *Brit. J. Exptl. Pathol.* **18**, 23–30.
Hyde, R. R. 1939. *Am. J. Hyg.* **30**, 47–55.
Hyde, R. R., and Gardner, R. E. 1939. *Am. J. Hyg.* **30**, 57–63.
Kilham, L., Herman, C. M., and Fisher, E. R. 1953. *Proc. Soc. Exptl. Biol. Med.* **82**, 298–301.
Ledingham, J. C. G. 1937. *Brit. J. Exptl. Pathol.* **18**, 436–449.
Nauck, E. G. 1937. *Zentr. Bakteriol. Parasitenk.* **140**, 160–163.
Paschen, E. 1936. *Zentr. Bakteriol. Parasitenk.* **138**, 1–14.
Schlesinger, M., and Andrewes, C. H. 1937. *J. Hyg.* **37**, 521–526.
Shope, R. E. 1932a. *J. Exptl. Med.* **56**, 793–802.
Shope, R. E. 1932b. *J. Exptl. Med.* **56**, 803–822.
Shope, R. E. 1936a. *J. Exptl. Med.* **63**, 33–41.
Shope, R. E. 1936b. *J. Exptl. Med.* **63**, 43–57.
Shope, R. E. 1936c. *J. Exptl. Med.* **63**, 173–178.
Shope, R. E. 1938. *Proc. Soc. Exptl. Biol. Med.* **38**, 86–89.
Smith, M. H. D. 1948. *Proc. Soc. Exptl. Biol. Med.* **69**, 189–193.
Smith, M. H. D. 1952. *Ann. N.Y. Acad. Sci.* **54**, 1141–1152.
Thompson, R. L. 1938. *J. Infectious Diseases* **62**, 307–312.

Milk Factor

Adelsberger, L. 1951. *Cancer Research* **2**, 658–662.
Andervont, H. B. 1941. *J. Natl. Cancer Inst.* **2**, 307–308.
Andervont, H. B. 1944. Unpublished, see Andervont, H. B. 1945.

Andervont, H. B. 1945. The Milk Influence in the Genesis of Mammary Tumors. A symposium on mammary tumors in mice, pp. 123–139. Moulton Ray Forest.
Andervont, H. B. 1952. *Ann. N.Y. Acad. Sci.* **54**, 1004–1011.
Andervont, H. B., and Bryan, R. W. 1944. Unpublished, see Andervont, H. B. 1945.
Andervont, H. B., and Bryan, R. W. 1944–1945. *J. Natl. Cancer Inst.* **5**, 143–150.
Andervont, H. B., and Dunn, T. B. 1948a. *J. Natl. Cancer Inst.* **8**, 227–234.
Andervont, H. B., and Dunn, T. B. 1948b. *J. Natl. Cancer Inst.* **9**, 89–104.
Andervont, H. B., and Dunn, T. B. 1950. *J. Natl. Cancer Inst.* **10**, 895–926.
Andervont, H. B., and McEleney, W. J. 1939. *Public Health Repts. (U.S.)* **54**, 1597–1603.
Andervont, H. B., Shimkin, M. B., and Bryan, W. R. 1942. *J. Natl. Cancer Inst.* **3**, 309–318.
Armstrong, E. C. 1948. *Brit. J. Cancer* **2**, 59–69.
Barnum, C. P., Ball, Z. B., and Bittner, J. J. 1946. *Cancer Research* **6**, 499.
Barnum, C. P., Ball, Z. B., and Bittner, J. J. 1947. *Cancer Research* **7**, 522–528.
Barnum, C. P., and Huseby, R. A. 1950. *Cancer Research* **10**, 523–529.
Barrett, M., and Deringer, M. K. 1952. *Cancer Research* **12**, 246.
Barrett, M., Deringer, M. K., and Dunn, T. B. 1952. *J. Natl. Cancer Inst.* **13**, 109–120.
Begg, A. M. 1949. *Brit. J. Cancer* **3**, 88–89.
Bennison, B. E. 1949. *J. Natl. Cancer Inst.* **10**, 175–178.
Bittner, J. J. 1936. *Science* **84**, 162.
Bittner, J. J. 1939. *Public Health Repts. (U.S.)* **54**, 1827–1831.
Bittner, J. J. 1942. *Cancer Research* **2**, 710–721.
Bittner, J. J. 1943. *Cancer Research* **3**, 441–447.
Bittner, J. J. 1944a. See Andervont, H. B. 1945.
Bittner, J. J. 1944b. *Cancer Research* **4**, 159–167.
Bittner, J. J. 1945. *Am. Assoc. Advance. Sci. Research Conf. on Cancer*, pp. 63–96. Moulton Ray Forest.
Bittner, J. J. 1948a. *Acta Unio Intern. contra Cancrum* **6**, 175–178.
Bittner, J. J. 1948b. *Proc. Soc. Exptl. Biol. Med.* **67**, 219–221.
Bittner, J. J. 1948c. *Cancer Research* **8**, 625–640.
Bittner, J. J. 1950. *Cancer Research* **10**, 204.
Bittner, J. J. 1952. *Cancer Research* **12**, 249, 387–398, 510–515, 594–601.
Bittner, J. J., Evans, C. A., and Green, R. G. 1945. *Science* **101**, 95–97.
Bittner, J. J., and Imagawa, D. T. 1950. *Cancer Research* **10**, 739–750.
Bonser, G. M. 1945. *J. Pathol. Bacteriol.* **57**, 413–422.
Bryan, W. R., Kahler, H., Shimkin, M. B., and Andervont, H. B. 1942. *J. Natl. Cancer Inst.* **2**, 451–455.
Dmochowski, L. 1945. *Brit. J. Exptl. Pathol.* **26**, 192–197.
Dmochowski, L. 1946. *Brit. J. Exptl. Pathol.* **27**, 391–393.
Dmochowski, L. 1948. *Brit. J. Cancer* **2**, 94–102.
Dmochowski, L. 1949a. *Brit. J. Cancer* **3**, 246–248.
Dmochowski, L. 1949b. *Brit. J. Cancer* **3**, 525–533.
Dmochowski, L. 1951. *Acta Unio Intern. contra Cancrum* **7**, 230–233.
Dmochowski, L. 1952. *Brit. J. Cancer* **6**, 249–253.
Dmochowski, L., and Orr, J. W. 1949. *Brit. J. Cancer* **3**, 520–524.
Dmochowski, L., and Passey, R. D. 1952. *Ann. N.Y. Acad. Sci.* **54**, 1036–1066.
Dmochowski, L., and Stickland, L. H. 1950. Quoted by Dmochowski, L., and Passey, R. D. 1952.
Fekete, E., and Little, C. C. 1942. *Cancer Research* **2**, 525–530.

Fekete, E., Little, C. C., and Richardson, F. L. 1952. *Cancer Research* **12,** 219–221.
Foulds, L. 1949. *Brit. J. Cancer* **3,** 230–239.
Gorer, P. A., and Law, L. W. 1949. *Brit. J. Cancer* **3,** 90–93.
Graff, S., Moore, D. H., Stanley, W. M., Randall, H. T., and Haagensen, C. D. 1948. *Acta Unio Intern. contra Cancrum* **6,** 191–196.
Graff, S., Moore, D. H., Stanley, W. M., Randall, H. T., and Haagensen, C. D. 1949. *Cancer* **2,** 755–762.
Graff, S., Randall, H. T., Carpenter, G. E., and Haagensen, C. D. 1946. *Science* **104,** 289.
Green, R. G., Moosey, M. M., and Bittner, J. J. 1946. *Proc. Soc. Exptl. Biol. Med.* **61,** 115–117.
Heidelberger, M., Graff, S., and Haagensen, C. D. 1952. *J. Exptl. Med.* **95,** 333–340.
Heston, W. E., and Deringer, M. K. 1952a. *J. Natl. Cancer Inst.* **13,** 167–175.
Heston, W. E., and Deringer, M. K. 1925b. *Cancer Research* **2,** 270.
Hummel, K. P., and Little, C. C. 1949. *Cancer Research* **9,** 137–138.
Huseby, R. A. Quoted by Bittner, J. J. 1945.
Huseby, R. A., Barnum, C. P., and Bittner, J. J. 1950. *Cancer Research* **10,** 516–520.
Imagawa, D., Bittner, J. J., and Syverton, J. T. 1950. *Cancer Research* **10,** 226–227.
Imagawa, D. T., Green, R. G., and Halvorson, H. O. 1948. *Proc. Soc. Exptl. Biol. Med.* **68,** 162–166.
Kahler, H., and Bryan, W. R. 1943. *J. Natl. Cancer Inst.* **4,** 37–46.
Kirschbaum, A., and Bittner, J. J. 1945. *Proc. Soc. Exptl. Biol. Med.* **58,** 18–19.
Korteweg, R. 1936. *Genetics* **18,** 350–362.
Lathrop, A. E. C., and Loeb, L. 1918. *J. Exptl. Med.* **28,** 475–500.
Little, C. C. 1936. *Am. J. Cancer* **27,** 551–555.
MacDowell, E. C., and Richter, M. N. 1935. *Arch. Pathol.* **20,** 709–724.
Malingren, R. A., and Law, L. W. 1951. *Cancer Research* **11,** 697–699.
Miller, E. W., and Pybus, F. C. 1945a. *Cancer Research* **5,** 84–93.
Miller, E. W., and Pybus, F. C. 1945b. *Cancer Research* **5,** 94–101.
Mühlbock, O. 1950a. *J. Natl. Cancer Inst.* **10,** 861–864.
Mühlbock, O. 1950b. *J. Natl. Cancer Inst.* **10,** 1259–1262.
Mühlbock, O. 1952a. *J. Natl. Cancer Inst.* **12,** 819–837.
Mühlbock, O. 1952b. *Klin. Wochschr.* **30,** 241–244.
Mühlbock, O., van Ebbenhorst, O., Teugbergen, W., and van Rijssel, T. G. 1952. *J. Natl. Cancer Inst.* **13,** 505–532.
Passey, R. D., Dmochowski, L., Astbury, W. T., and Reed, R. 1947. *Nature* **160,** 565.
Passey, R. D., Dmochowski, L., Astbury, W. T., Reed, R., and Johnson, P. 1950. *Nature* **165,** 107.
Passey, R. D., Dmochowski, L., Astbury, W. T., Reed, R., and Johnson, P. 1951. *Acta Unio Intern. contra Cancrum* **7,** 299–303.
Porter, K. R., and Thompson, H. P. 1948. *J. Exptl. Med.* **88,** 15–23.
Porter, K. R., and Thompson, H. P. 1949. *Cancer Research* **9,** 557–558.
Prehn, R. T. 1952. *J. Natl. Cancer Inst.* **12,** 1127–1140.
Pullinger, B. D. 1947. *Brit. J. Cancer* **1,** 177–191.
Pullinger, B. D. 1952. *Brit. J. Cancer* **6,** 69–77, 78–79.
Rudali, J. 1952. *Compt. rend. soc. biol.* **146,** 916–918.
Shimkin, M. B. 1943. See Andervont, H. B. 1945.
Shimkin, M. B., and Andervont, H. B. 1945. *Am. Assoc. Advance. Sci. Research Conf. on Cancer,* pp. 97–105. Moulton Ray Forest.
Shimkin, M. B., Grady, H. G., and Andervont, H. B. 1941. *J. Natl. Cancer Inst.* **2,** 65–80.

Smith, F. W. 1945. *Science* **101**, 279–281.
Taylor, A., and Carmichael, N. 1949. *Cancer Research* **9**, 498–503.
Taylor, A., Carmichael, N., and Norris, T. 1948. *Cancer Research* **8**, 264–268.
Taylor, A., and Kynette, A. 1945. *Am. Assoc. Advance. Sci. Research Conf. on Cancer*, pp. 24–39. Moulton Ray Forest.
Taylor, A., Thacker, J., and Pennington, D. 1942. *Science* **96**, 342–343.
Van Gulik, P. J., and Korteweg, R. 1940. *Koninkl. Ned. Akad. Wetenschap., Proc.* **43**, 891. Quoted by Bittner, J. J. 1945.
Visscher, M. B., Green, R. G., and Bittner, J. J. 1942. *Proc. Soc. Exptl. Biol. Med.* **49**, 94–96.
Woolley, G. W., Dickie, M. M., and Little, C. C. 1952. *Cancer Research* **12**, 142–152.
Woolley, G. W., Law, L. W., and Little, C. C. 1941. *Cancer Research* **1**, 955–956.

General Considerations

Altenburg, E. 1946. *Am. Naturalist* **80**, 559–567.
Anderson, T. F. 1946. *Cold Spring Harbor Symposia Quant. Biol.* **11**, 1–13.
Bernhard, W., Gautier, A., and Oberling, C. 1951. *Compt. rend. soc. biol.* **145**, 566–569.
Bernhard, W., Haguenau, F., Gautier, A., and Oberling, C. 1952. *Z. Zellforsch. u. mikroskop. Anat.* **37**, 281–300.
Bernhard, W., Haguenau, F., and Oberling, C. 1952. *Experientia* **8**, 58–59.
Biebl, R. 1935. *Protoplasma* **24**, 225–257.
Brachet, J. 1952. Le rôle des acides nucléiques dans la vie de la cellule et de l'embryon. Masson & Cie, Paris.
Butenandt, A. 1952. *Verhandl. deut. pathol. Ges.* **35**, 70–80.
Caspersson, A. 1950. Cell Growth and Cell Function. W. W. Norton & Co., New York.
Claude, A. 1943a. *Science* **97**, 451–456.
Claude, A. 1943b. *Biol. Symposia* **10**, 111–129.
Claude, A. 1946. *J. Exptl. Med.* **84**, 51–89.
Claude, A. 1947–1948. *Harvey Lectures* **43**, 121–164.
Claude, A., and Murphy, J. B. 1933. *Physiol. Revs.* **13**, 246–275.
Dalton, A. J. 1951. *Am. J. Anat.* **89**, 109–133.
Dalton, A. J., Kahler, H., Striebich, M. J., and Lloyd, B. 1950. *J. Natl. Cancer Inst.* **11**, 439–461.
Darlington, C. D. 1939. The Evolution of Genetic Systems. Cambridge University Press, Cambridge.
Darlington, C. D. 1948. *Brit. J. Cancer* **2**, 118–126.
Darlington, C. D., and Mather, K. 1949. Elements of Genetics. Allen and Unwin, London.
Du Buy, H. G., and Woods, M. W. 1943. *Phytopathology* **33**, 766–777.
Du Buy, H. G., and Woods, M. W. 1945. *Am. Assoc. Advance. Sci. Research Conf. on Cancer*, pp. 162–169.
Foulds, L. 1940. *Am. J. Cancer* **39**, 1–39.
Gautier, A., and Diomede-Fresa, V. 1953. *Mikroskopie* **8**, 23–31.
Graffi, A. 1940. *Z. Krebsforsch.* **49**, 477–495.
Graffi, A. 1941. *Z. Krebsforsch.* **50**, 501–551.
Gross, L. 1951. *Cancer* **4**, 626–633.
Haddow, A. 1944. *Nature* **154**, 194–199.
Haddow, A. 1947. *Brit. Med. Bull.* **4**, 331–341.
Horning, E. S., and Petrie, A. H. K. 1927. *Proc. Roy. Soc.* (*London*) **B102**, 188–206.
Kidd, J. G. 1938. *Proc. Soc. Exptl. Biol. Med.* **38**, 292–295.

Kidd, J. G. 1940. *J. Exptl. Med.* **71,** 335–371.
Kidd, J. G. 1946a. *Cold Spring Harbor. Symposia Quant. Biol.* **11,** 94–110.
Kidd, J. G. 1946b. *J. Exptl. Med.* **83,** 227–250.
Kidd, J. G. 1950. The Pathogenesis and Pathology of Virus Diseases. Columbia University Press, New York.
L'Héritier, Ph. 1948. *Heredity* **2,** 325–348.
Loeb, L. 1937. *Acta Unio Intern. contra Cancrum* **2,** 148–194.
Loeb, L. 1944. *Colloid Chem.* **5,** 995–1050.
von Moellendorff, W. 1939. *Z. Zellforsch. u. mikroskop. Anat.* **29,** 706–749.
Monné, L. 1948. *Advances in Enzymol.* **8,** 1–69.
Nadson, G. A., and Rochlin, E. J. 1933. *Protoplasma* **20,** 31–41.
Newcomer, E. H. 1940. *Botan. Rev.* **17,** 53–89.
Oberling, C., Bernhard, W., Fèbvre, H. L., and Harel, J. 1951. *Rev. hématol. (Paris)* **6,** 395–400.
O'Brien, J. M. 1942. *Am. J. Botany* **29,** 479–491.
Palade, G. E. 1952. *J. Exptl. Med.* **95,** 285–298.
Porter, K. R., Claude, A., and Fullam, E. F. 1945. *J. Exptl. Med.* **81,** 233–246.
Porter, K., and Thompson, H. P. 1947. *Cancer Research* **7,** 431–438.
Rhoades, M. M. 1946. *Cold Spring Harbor Symposia Quant. Biol.* **11,** 202–207.
Rhoades, M. M. 1949. Colloques internationaux du C.N.R.S., Vol. VIII, pp. 37–44. Editions du C.N.R.S., Paris.
Rhoads, C. P. 1949. The Chemistry and Physiology of Growth. Princeton University Press.
Sonneborn, T. M. 1943. *Proc. Natl. Acad. Sci. U.S.* **29,** 329–343.
Sonneborn, T. M. 1947. *Advances in Genet.* **1,** 263–358.
Smith, W. G., and Rous, P. 1945. *J. Exptl. Med.* **81,** 621–646.
Strong, L. 1949. *Yale J. Biol. Med.* **21,** 293–299.
Vago, C. 1951. *Rev. can. biol.* **10,** 299–308.
Woods, M. W., and Du Buy, H. G. 1941. *Phytopathology* **31,** 278–290.
Woods, M. W., and Du Buy, H. G. 1943. *Phytopathology* **33,** 637–655.
Zollinger, H. 1950. *Rev. hématol. (Paris)* **5,** 696–745.

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Experimental Cancer Chemotherapy

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CONTENTS

I. Introduction

As recently as 1945 experimental cancer chemotherapy was able to offer little encouragement for the palliation of cancer. Greenstein (1947) found it unnecessary to devote to cancer chemotherapy more than ten pages out of approximately thirty-five fold that number in his Biochemistry of Cancer. Considered worthy of emphasis therein were bacterial metabolites, benzene, arsenite, radioactive isotopes, and colchicine only.

Although the first human cancer chemotherapy cure remains to be found, numerous studies have advanced considerably the status of cancer chemotherapy in the light of the situation that existed ten or even a half dozen years ago. This, however, can hardly be a basis of satisfaction among investigators in view of the tremendous task to be done. Nevertheless, it will be worth while to review the developments that have revealed agents effective against cancer in animals and some which are of limited use in man. This survey will be concerned with studies in experimental cancer chemotherapy with only such references to clinical results, already reviewed by others (Karnofsky, 1948; Shimkin and Bierman, 1949b; Gellhorn and Jones, 1949; Karnofsky and Burchenal, 1950; Karnofsky, 1952; Gellhorn, 1953), as are desirable to show the fate of the compounds when they have been subjected to the ultimate, practical challenge of clinical trial.

The reviewer will take advantage of the latitude provided by this new series of Advances to choose to cover the studies of the postwar years. It is

during this period that experimental cancer chemotherapy may be said to have come of age, though early references to the subject go back at least to the middle of the 14th century (Dyer, 1949). The assumption of a more respectable status has resulted perhaps both from the impact of developments during World War II and from the generous public support given to cancer research in recent years with the consequent attraction of varied talent to the field. Contributing factors from the years 1940–45 were the concept of group efforts in large scale attacks upon problems, the first successful antibiotics with their new order of effectiveness, and compounds such as the nitrogen mustards to revitalize thinking in experimental cancer chemotherapy. In presenting a rounded picture of recent developments it will be clear that not all the material included can be construed as representing advances.

II. Test Methods

Without attempting an extensive historical accounting of the background, it may be of value to discuss test methods representative of those that have been used to find and further study the antitumor activity of compounds.

1. In vitro *Tests*

After acquaintance with the laborious procedures required to test materials for *in vivo* antitumor activity, there probably are few investigators in the field who have not sought in vain for a satisfactory short-cut procedure. In establishing a screening program to survey large numbers of compounds, it must be made as simple and easy to conduct as possible, but simplicity must not be achieved at the expense of lost significance in the data. Thus far the *in vitro* procedures do not appear to have yielded data of value for subsequent *in vivo* studies. The lack of *in vivo* usefulness of *in vitro* data seems to be true whether the *in vitro* tests are based upon cytological evaluations (Cohen *et al.*, 1947), respiration measurements (Wade *et al.*, 1951), or bioassays of the exposed tumor material (Sugiura, 1938). Assays utilizing microorganisms or microbiological techniques offer tempting biological test systems, but there is no indication of the general usefulness of any of these procedures. Nevertheless there are examples of the value of testing any material that shows a marked influence upon any microorganism (Kidder *et al.*, 1949; Clarke *et al.*, 1952).

2. Tissue Culture

This form of an *in vitro* study provides comparison of effects of materials upon both normal and cancer cells in an environment that permits some cell growth. The procedure has both the advantages and disadvan-

tages that accrue from its conduct in the absence of the intact animal with its uncertain influences for modification, complete destruction, excretion, or unusual distribution of the test compound. It has been possible by this technique to find certain antitumor properties in materials from antibiotic preparations (Cornman, 1944a,b), plants (Ormsbee *et al.*, 1947), alkaloids, acridines, and various other materials (Lettré, 1951). These substances have shown toxicities greater for cancer cells than normal tissue cells. Biesele *et al.* (1952a) have employed a modification of the procedure used by Ormsbee *et al.* (1947). In brief it is as follows: Along the inner wall of the lower third of test tubes, 16 × 150 mm., six explants of mouse sarcoma 180 about 1–2 mm. in diameter are placed in a row. On the opposite inner wall of the tube are placed 6 explants of abdominal skin of AKR mouse embryo. All the tissue explants are held in place by thin clots of chicken plasma. One milliliter of nutrient solution, containing Gey's salt solution, chick embryo extract, human placental serum, horse serum, and small amounts of penicillin G and streptomycin, is added. The tubes are incubated at 37°C. for 24 hours in a rotating drum (10 rph). After satisfactory growth, 0.1 ml. of the solution (or suspension) of test material is added as replacement of an equal volume of nutrient. Two or three tubes are set up at each selected level ranging from 4.0 to 0.125 micromoles/ml. After an additional 24-hour incubation, the extent of damage to the cultures is evaluated based on extent of growth, and degrees of rounding up, granularity, and disintegration of cells.

By this technique there has been demonstrated a striking differential damage from 2,6-diaminopurine on mouse sarcoma compared with mouse embryo epithelium (Biesele *et al.*, 1951); however, the reverse was true when corresponding rat tissues were employed. Other 2-substituted adenines also have given differential damage. With the exception of purine riboside the purine nucleosides examined have not produced any differential damage (Biesele *et al.*, 1952a and unpublished data). The ability of adenine to antagonize some of the toxic purines, including 2,6-diaminopurine, was established (Biesele *et al.*, 1952b). Adenine was capable, also, of blocking the differential toxicity of 2-aza-adenine (Biesele, 1952).

3. Egg Culture

Another system which, while highly artificial, is capable of yielding useful information is that employing the chick embryo for growth of animal tumors on the chorioallantoic membrane of the yolk sac. This technique represents one of the oldest used for heterologous growth (Murphy, 1913). Campbell (1949) has studied such growth with special interest in the associated ectodermal lesions of the membrane. Karnofsky *et al.* (1947a) have used it to demonstrate activity of nitrogen mustard-

like compounds against tumors. Karnofsky's technique is as follows: A window is cut in the shell over the vascular area of the membrane of an 8-day embryo. A tumor fragment 1–2 mm. in diameter is placed on a large blood vessel and the shell opening sealed with Scotch tape or a coverglass and paraffin. The egg is incubated for 4 days at 99–100°C. and then the largest dose of material tolerated by the embryo is injected into the yolk sac. Five days later the egg is opened and the tumor is removed for bioassay in mice and histological evaluation of damage.

Determination of the toxicity of agents for the chick embryo in the above procedure led quite naturally to observations of striking localized damage to the embryo (Ridgway and Karnofsky, 1952). It is not within the scope of this discussion to do more than recognize the possibility that substances capable of producing such marked, specific damage to normal embryo development may prove worth consideration for any possible action against abnormal cells.

Taylor *et al.* (1942, 1943, 1948) and Taylor (1950) have described a procedure for the cultivation of mouse and rat tumors in the yolk sac of the embryonated egg. The procedure including a chemotherapy trial is as follows: 0.4 cc. of 1:8 suspension of tumor, e.g., a C3H mammary adenocarcinoma, in saline is injected into the yolk sac of a fertile egg incubated for 4 days. After tumor implantation the eggs are further incubated 8 days. At the end of that time the eggs are kept for a few hours or overnight in a position so that the embryo and chorioallantoic membranes are located in the dorsal area of the egg. Eggs are separated into control and experimental groups of 18 to 20 each. The shell over the blood vessels of the chorioallantoic membrane is dented with a blunt scissor point and 0.1 to 0.2 cc. of the test chemical injected by 22 or 23 gage needle and syringe into the space between the shell and chorioallantoic membranes. Sometimes yolk sac injection of the test chemical is employed. The tumors and embryo may be harvested either 24 or 48 hours after injection of test material for evaluation of effect based upon comparative weights of embryo and tumors of the treated and control groups. Observations of any significant gross or microscopic morphologic changes are also made. By this technique it has been possible to demonstrate a differential inhibition of growth greater for the tumor than for the embryo with aminopterin and with several extracts of *Cooperia pedunculata Herb* (Rain Lily) (Taylor *et al.*, 1951).

4. Cytological Tests

Probably the antitumor screening program which has been in operation for the longest period is that of Shear *et al.* (1947). This program has now tested approximately 3000 compounds by a technique which em-

ploys gross and histological observation of damage to sarcoma 37. The test has been conducted as follows: Mouse sarcoma 37 is implanted intramuscularly. When the tumor is one week old, a single subcutaneous injection of test compound is made into the flank contralateral to the tumor. The dose selected is less than an LD_{10}. Mice from each test group are sacrificed at intervals of 4, 24, and 48 hours after injection of test chemical. At the time of sacrifice the tumors are examined grossly for evidence of damage and sections prepared for microscopic study of possible tumor damage. Normal tissue is included for control purposes. For those compounds showing capacity to damage sarcoma 37 additional studies naturally are scheduled including repeated injections at maximum tolerated doses in normal and sarcoma 37 bearing mice and against other types of experimental tumors. Toxicologic data are obtained in other species. This cytological technique has revealed the tumor damaging action of certain arsenicals (Leiter *et al.*, 1952a), colchicine and related compounds (Leiter *et al.*, 1952b,c, 1953), diphenylethylamines and podophyllin and its components, podophyllotoxin, α and β peltatins (Hartwell and Shear, 1947). These compounds are now being studied for their mechanisms of action. Most of them have not received clinical trial.

Another cytological study is that of (Koller, 1949). It is not proposed as a routine screening test, but it would appear to be of probable use in the detection of substances which act as mutagenic agencies.

5. Cytocidal Test

This represents a quick method of determining under physiologic conditions the relative susceptibility of abnormal cells to any selected material compared with that of the most sensitive system of the host which may determine the acute lethal dose. Although never considered a therapeutic procedure nor even a practical screening device, the test may develop useful information. Through indication of the rapidity with which a compound destroys the viability of tumor cells *in vivo* an indication of the mode of action may be provided. Further experience with the test may indicate that it is capable of predicting whether an agent is likely to be only palliative and to what extent it has a useful dose range. An example of such a technique is one testing the sterilization of leukemic cells *in vivo* (Burchenal *et al.*, 1951a).

In the *in vivo* sterilization test AKR mice have received intraperitoneal injections of leukemic cells. Between 8 to 14 days after the transplantation of leukemia, depending upon the strain of leukemia, test materials have been injected intraperitoneally into groups of 2 mice each for dose levels starting at the LD_{50} and increased by 100% from each group to the next higher one. Two hours after the injection, and after such other time intervals as might be indicated, the mice have been sacrificed and ex-

amined for manifestations of leukemia. Then preparations of leukemic cells from spleen (or localized tumor) of the treated mice have been injected into four recipient mice for each test group. These conditions have provided data for determining the dose level (if any) capable of sterilizing leukemic cells *in vivo*. In this study some nitrogen mustards definitely show a sterilizing effect upon leukemic cells whereas 2,6-diaminopurine and some 4-amino analogs of folic acid have been negative even at doses 50 to 100 times the LD_{50}.

6. Mouse Leukemia

Flory *et al.* (1943) developed a method of testing the effectiveness of compounds against leukemia in mice. Subsequently other investigators

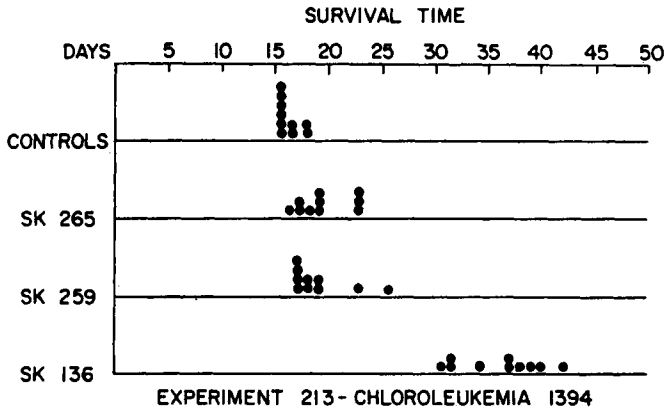


FIG. 1. Chart showing increase in survival of leukemic mice treated with a nitrogen mustard, SK 136. Each dot represents the death of a mouse with leukemia. SK 265 and 259 are ineffective compounds. (Courtesy of J. H. Burchenal.)

have employed similar procedures (Law, 1947; Burchenal *et al.*, 1948a; Kirschbaum *et al.*, 1948). In general, the experimental leukemia chemotherapy studies have been conducted in mice of an inbred strain which have been given leukemia by intraperitoneal injections of a suspension containing a known number of leukemic cells. The cells have been obtained from a minced spleen of a mouse acutely ill with transmitted leukemia. Starting 24 to 48 hours after injection of the leukemic cells, injections of test material usually have been made intraperitoneally, every day or two at the maximum dose tolerated on the repeated basis. After the course of injections the survival time of the treated mice has been compared with that of the untreated controls. In some instances the beneficial compounds, such as the nitrogen mustards and the folic acid analogs, have increased survival time from 100 to 200% beyond that of the controls, Fig. 1.

7. *Solid Tumors*

A. *Screening.* The most extensive and most numerous studies seeking cancer chemotherapeutic agents have been conducted using solid tumors *in vivo* as the test object. The conditions of these tests have varied extensively as to (1) selection of the host and the tumor, (2) method of administration of the agents including variables of route, frequency, dose level, and time of initiation with respect to age of tumor and (3) final evaluation of effect, whether it be failure of tumor implants to grow, inhibition of growth during a selected time period, increased regression rate of tumors, increased survival time of the host, or cytological criteria already mentioned.

Many solid tumors are available for which there is considerable information (Dunham and Stewart, 1953). For most of these there is little or no chemotherapy experience; however, if for pertinent reasons one of them is elected for use in chemotherapy, it can be determined at least that the tumor is not unduly sensitive or resistant by test with a number of demonstrated tumor inhibitory substances. After selection of the tumor the test conditions can be decided upon with the hope that continued testing will indicate wisdom in the arbitrary choices made for the screening program. Although certain techniques can be discredited readily, e.g., those involving intratumoral or pretumor implantation injections, some of the considerations may be illustrated best by discussing several solid tumor screening procedures which have been used to screen a considerable series of compounds.

Haddow and Robinson (1937), in testing carcinogens for tumor inhibitory activity, have described use of the Walker rat carcinoma 256. In this technique 2–3 mm. cubes from 10- to 14-day-old donor tumors are transplanted by cannula subcutaneously through a small incision in the rat's skin. Compounds are injected intraperitoneally, two injections within three days after tumor implantation. Other dosage schedules may be employed. Controls receive injections of the solvent or suspending medium. The tumors are evaluated by weighing on removal from the hosts three weeks after implantation. Blood counts are taken and histology of tumor and normal tissues studied. This technique has revealed antitumor properties of carcinogenic hydrocarbons, of urethane, of the nitrogen mustards and related compounds including ethylenimines, and esters of sulfonic acid. (See references later under specific compounds.)

Walpole (1951) and associates have used the Walker carcinoma 256 with some modifications in experimental details of Haddow's procedure. Donor rats 8–12 days old provide 200–300 mg. transplants for subcutaneous implantation by trochar into rats 90–120 g. in weight. Rats in groups

of 10–15 each, matched for sex distribution and body weight, are injected intraperitoneally daily for 10–12 days starting 24 hours after tumor implantation. On the fourteenth to fifteenth post-transplant day the tumors are dissected out and weighed. Evaluations are made on the tumors in the heavier half of each group. Inhibitions of growth are considered significant only when the tumors of the treated group weigh half or less than those in the control group. In studies of Hendry *et al.* (1951a,b,c) a number of methylolamines, bis-epoxides, and ethylenimines were found active.

In 1946, Lewis *et al.* found oral administration of the oxazine dye, Nile Blue A, to tumor-bearing mice resulted in staining the tumors and retarding their growth. Subsequently additional dyes and other tumors were tested by this technique (Lewis *et al.*, 1949). The test is conducted as follows: The compounds are administered by mixing in the food at a concentration of 0.2 to 0.4% depending upon toxicity. Various strains of mice and rats with different tumors have been used. The feeding of dyes is started 1 to 2 hours after tumor implantation and the animals are sacrificed 14 to 15 days later. Tumor inhibition is considered to have occurred when the calculated tumor volumes are less than 1000 cu. mm. with the untreated controls averaging 9600 cu. mm. It is important in considering this test procedure to note that it was among the first to demonstrate an activity of 2,4,6-tris(ethylenimino)-s-triazine and further that it indicated its probable activity by the oral route (Lewis and Crossley, 1950).

In a program utilizing mouse sarcoma 180 approximately 9500 compounds and many materials of natural origin have been tested for their ability to retard the growth of the tumor. Sarcoma 180 was chosen because of its nearly 100% transplantability, low regression rate, rapid growth, lack of host strain requirement, and its apparent intermediate sensitivity to several adverse agents. The test has been conducted as follows (Stock and Rhoads, 1949): Mice approximately 20 g. are weighed at beginning and end of the treatment. Tumor fragments, approximately 1- to 2-mm. cubes, are transplanted subcutaneously by trochar into the axillary region. Twenty-four hours later compounds in maximum tolerated doses on a repeated basis, are injected intraperitoneally twice daily for seven days. On the eighth day the tumors are measured in two diameters by calipers. Any test material failing to hold the tumor growth to below three-quarters the average diameter of the controls is considered negative. Twelve compounds have given a maximum grading (no growth of tumors to that of one-quarter the average diameter of the controls). The compounds include seven of the 4-amino folic acid analogs, a nitrogen mustard, TEM (2,4,6-tris(ethylenimino)-s-triazine), and three phosphoramides. The technique is similar to that of Lazlo and Leuchtenberger (1943a) although they used a shorter time period for evaluation. Another screening program has used

TABLE I
Differences in Inhibition of Various Tumors by Selected Compounds

Compound Name	Dose Level, mg./k./day	Mouse Tumors					Rat Tumors		
		S180	EO 771	HP melanoma	Wagner osteogenic sarcoma	Patterson lymphosarcoma	Sarcoma 39	F-J carcinoma	Walker 256
8-Azaguanine	75	−	++	−	−	−	−	−	−
2,4,6,tris(ethylen-imino)-s-triazine	0.25	±	−	−	+	−	+++	+++	+++
Aminopterin	0.25	++	+	±	−	++	+++	−	+++
Methoxypyridoxyl bis(β-chlorethyl) amine	5.0	±	±	−	++	±	+++	+++	+++
Cortisone acetate	37.5	−	+	−*	++	++	−	−	−

Note. Grading of tumor inhibition:
− Growth more than ¾ the average diameter of the controls.
± Growth from ½ to ¾ the average diameter of the controls.
+ Growth from ¼ to ½ the average diameter of the controls.
++ No growth or growth to ¼ average diameter of the controls
+++ Destruction of tumor.
* Negative at 62.5 mg./k./day.

sarcoma 180 with a technique similar to that described above (Goldin *et al.*, 1949b). Thus far the sarcoma 180 test has not appeared suitable for using survival time as a criterion of effectiveness though an unusually effective compound should show up well. Confidence in the sarcoma 180 test arises from the fact that all 12 of the best compounds from those surveyed, though not curative, have shown benefits in certain forms of the human disease. It is recognized that one tumor and one test alone may miss useful compounds and for this reason tests in a tumor spectrum are considered advisable.

B. *Solid Tumor Spectrum.* The use of a group of tumors, varied as to cell type, host species or strain, rate of growth and implied biochemical differences, has been stressed as a desirable if not essential part of any extensive chemotherapy program (Bauer, 1949; Stock, 1950), whether within one laboratory or as part of a cooperative program among a number of laboratories. The value or need of a tumor spectrum is predicated upon belief in biochemical differences in the metabolism of different tumors. Although earlier studies tended to support belief in the similarity of metabolism among cancer cells (Greenstein, 1947), various experimental and clinical observations now indicate there are differences in responses of tumors to chemotherapeutic agents (Table I) which may reflect biochemical differences (Hirschberg *et al.*, 1952). The varied responses of the tumors to the agents listed in the table suggest the advisability of testing theoretically interesting compounds with a variety of tumors and testing against all forms of human cancer those compounds found active against animal tumors. It is too early to say whether a spectrum of animal tumors will be useful in suggesting which types of human cancer should be studied first. A value of the spectrum will be in providing tools for biochemical studies, e.g., the marked difference in response of two rat tumors to aminopterin may permit a better understanding of the mechanism of action of the folic acid analogs.

8. Tumors in Ascitic Form

In the past few years use of the ascitic form of tumors has gained in popularity. Studies on the Yoshida sarcoma are said to have occupied approximately 30% of the recent cancer research effort in Japan (Nakahara, 1952), and this would appear supported by the literature. It seems quite likely that an increased number of tumors will be developed in this form (Klein and Klein, 1951). Klein (1951b) has advocated the ascites tumor as a useful tool for quantitative studies on growth and on the biochemistry of neoplastic cells and with his associates has studied the nucleic acid content of ascites tumor cells (Goldberg *et al.*, 1950a). The ascites tumor permits determinations of the effect of chemotherapeutic agents

by daily biopsies in the form of aspirated ascitic fluid. The large amount of ascites produced may provide an additional method of evaluation by weight measurement of the whole animal (Lettré, 1950; Sugiura and

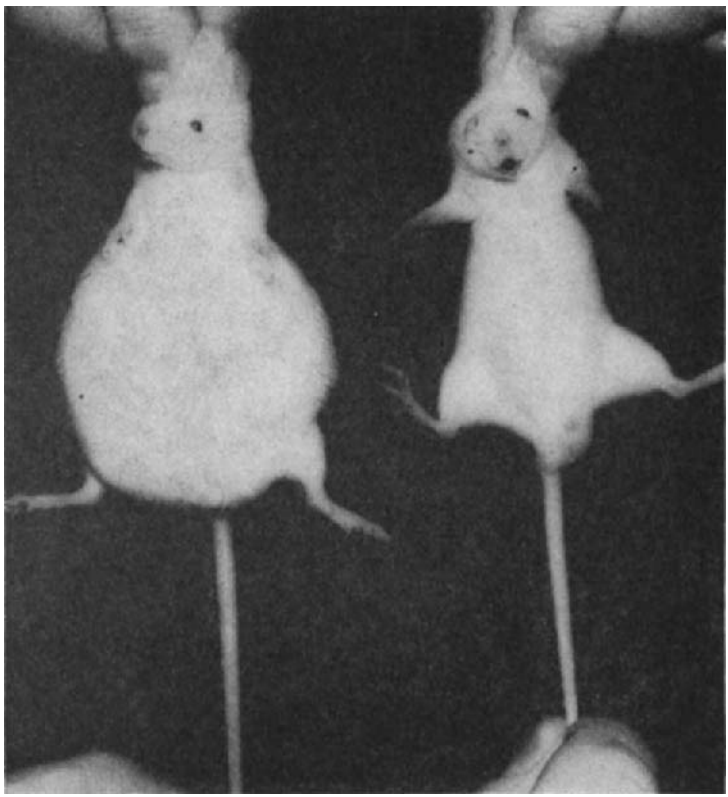


FIG. 2. Comparison of mouse (right) treated for seven days with subcutaneous injections of cortisone acetate 37.5 mg./k./day with untreated control (left). The animals were photographed 7 days after treatment ended and 14 days after injection with fresh ascitic fluid containing one million cancer cells. (Courtesy of K. Sugiura.)

Stock, 1951) (Fig. 2); Klein (1951a), however, has questioned the reliability of this method because he has not observed sufficiently uniform development of ascites in his experience nor necessarily a decrease in number of cells with decreased ascites following treatments. Increase in survival time represents another possible measure of the effectiveness of therapeutic trials inasmuch as the animals with certain inoculation doses of tumor cells and under controlled conditions follow a fairly uniform course. Yoshida (1952) has reported that methyl bis(β-chlorethylamine)-oxide is effective against the Yoshida sarcoma.

Sugiura and Stock (1951) have reported tests of a number of standard antitumor substances for their effects by subcutaneous and intraperitoneal routes against the ascitic form of the Ehrlich carcinoma. With the exception of cortisone, which was most effective subcutaneously, the only compounds active against the tumor were TEM and two nitrogen mustards. It is not surprising that these were more damaging to the ascitic tumor cells when given intraperitoneally for the resulting intimate mixture of ascitic cells and test agent is likely to provide less of a challenge to the test material than its trial by another route or against a solid tumor. Such a test on the ascitic tumor is in essence an *in vivo in vitro* test.

9. Heterologous Growth

Developments in the growth of tumors in species other than that of origin have been of definite theoretical interest with possible practical applications. One use will be mentioned with respect to the virus studies (Toolan and Moore, 1952). As in the virus studies, heterologous growth has its roots in much earlier studies dating back at least to 1912 (Murphy, 1912, 1913; Hegner, 1913).

Murphy's studies indicated the possibility for growth of tumors in heterologous species when the embryo was employed, for example, the chick embryo. Reference has been made to Karnofsky's use of the technique (Karnofsky *et al.*, 1947a). In his experience human tumors transplanted directly to the chick embryo, did not grow well. Sommers *et al.*, (1952b) have reported survival only of some human tumors transplanted to the chorioallantoic membrane. Recently, however, Toolan (1952) has observed that human cancer will grow well on the membrane of the chick embryo after the neoplastic tissue has been passed through the rat. Hegner (1913) revealed the prospects for growth in a different species by use of a special site, the anterior chamber of the eye where immunological factors are not a problem. He demonstrated that mouse tumors would grow in the eyes of rats, rabbits, and guinea pigs. Limited success was attained with human tumors.

The studies of Greene (1941, 1951), Greene and Lund (1944), Greene and Murphy (1945) stimulated reconsideration of heterologous growth and a number of experimentalists re-examined the problem, in some instances with the objective of providing improved human test material for chemotherapy experiments. Dyer and Kelly (1946) investigated the cultivation of tumors in the anterior chamber of the guinea pig's eye. Included were tumors from mouse, rat, guinea pig, and human. It was concluded from this study that large numbers of guinea pigs would be required to insure growth of human tumor grafts and that even at maximum growth only small amounts of tumor material could be produced; therefore, Dyer

and Kelly thought the procedure would be of very doubtful practical value. The use of the anterior chamber of the rabbit's eye for cultivation of the Brown-Pearce carcinoma has been advanced as a possible screening technique with the intriguing suggestion that it could permit simultaneous studies of pharmacology in a larger laboratory animal (Shapiro *et al.*, 1950). Browning's studies (1949) provided information on the ability of normal mouse tissues of different cell types and different stages of development to grow in rats and various strains of mice. Patti and Moore (1950) found that mouse sarcoma 180 would grow quite well in newborn rats while the tumors failed to take or regressed within a short period in older animals. In adult hamsters, however, the transplants grew. There was some ability of this tumor and a methylcholanthrene induced mouse sarcoma to grow in successive intraperitoneal passage in adult hamsters. The possibility of using this technique for human tumors was suggested (Patti and Biesele, 1951). Patt *et al.* (1952) have reported successful use of the cheek pouch of the hamster for heterologous tumor growth including neoplasms from man. Chute *et al.* (1952), after successful use of the hamster's cheek pouch for implants of human cancer tissue, concluded the method is relatively simple and appears to be of great potential value.

Thus far, the most promising prospect of heterologous growth of human tumors for utilization in chemotherapy studies appears to be that of Toolan (1951a). Following the lead of Murphy (1914) with mouse tumors, she has developed a technique for the growth of human tumors in x-irradiated rats and mice. Though initially many implants failed to show mitoses or capacity for retransplantation, evidence was obtained that successive transplantations could be achieved. Further studies have improved the successful implants so that 75% of the epidermoid tumors become vascularized and grow for a time. See Fig. 3. It was also observed in other studies that autonomy of the tissue implanted was not an essential for successful transplantation because normal adult human epithelium implanted subcutaneously in x-irradiated hamsters and rats became vascularized, proliferated, and increased for about two weeks before sudden death of the implants (Toolan, 1951b). Sufficient success was obtained with implants of human cancer into irradiated rats that Sommers *et al.* (1952a) have considered it a technique suitable for wider use. Recently these developments have been extended by preliminary observations of limited success in the growth of human tumors in cortisonized rats and hamsters (Toolan, 1953; Hoch-Ligeti and Hsu, 1953). These results recall those of Foley (1952), who found that cortisone made possible the successful transplantation of certain strain specific mouse tumors into alien strains of mice.

This technique for growth of human tumors will require intensive de-

velopment before it can be used as a practical tool on a large scale. Additional factors influencing transplantation must be elaborated and, if possible, methods for securing a more extensive proliferation and uniformity of successful implantations in order that the results of any chemotherapeutic procedures will be subjected to a minimum of uncertainties inherent in this unusual technique. Among the considerations are proper

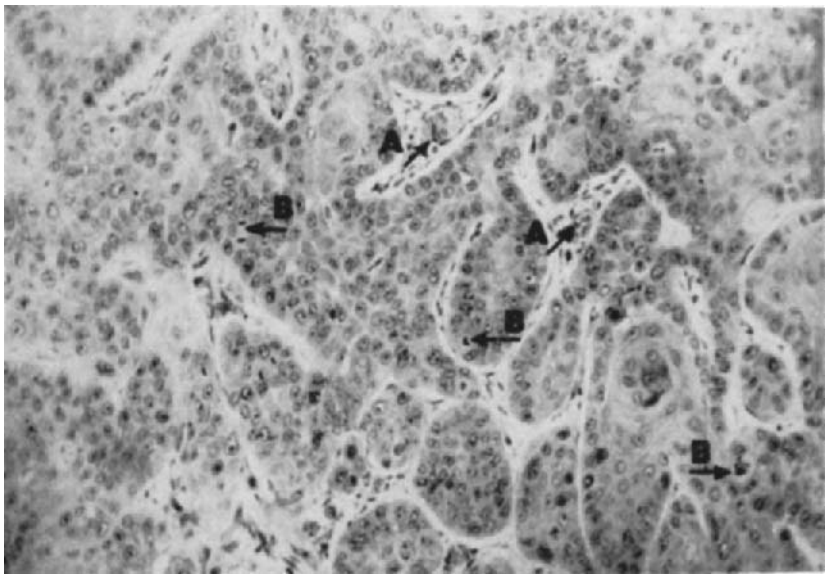


FIG. 3. Human epidermoid carcinoma after 2 generations (26 days) in the subcutaneous tissues of x-irradiated rats. Original implantation, a tissue mince. Arrows A point to blood vessels and arrows B to dividing cells. ×266. (Courtesy of H. W. Toolan.)

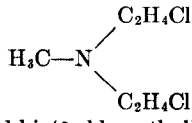
x-ray or cortisone dosage and individual variations in response with the resultant problems of early terminations of the experiments by host deaths or recoveries with attendant immunological influence upon the heterologous growth. The difficulties may be so serious as to discourage serious attempts to overcome them; however, the technique may represent a sufficiently good compromise between tests in mouse and man as to justify adequate effort to try to forge it into a useful tool.

III. MATERIALS TESTED

1. Synthetic Chemicals

A. *Reactive Compounds.* (a) *Nitrogen mustards.* So much has been written about the nitrogen mustards that any discussion of them now can-

not fail to be repetitious nor can they be adequately discussed in a few paragraphs. Nevertheless, as they, along with the steroids, have provided so much stimulus to renewed efforts and hope in cancer chemotherapy, it is impossible to omit them from anything that attempts coverage of recent advances in cancer chemotherapy. Furthermore the mustards have sired several groups of related compounds, the ethylenimines, the epoxides, and the esters of sulfonic acids. The historical background has been reviewed by Gellhorn and Jones (1949), and it has also been included in reviews of the numerous chemical reactions and biological effects produced by the nitrogen mustards (Gilman and Philips, 1946; Philips, 1950). The extensive studies of the sulfur and nitrogen mustards in mammals revealed their leukopenic action. This led to tests of the nitrogen mustards against



Methyl bis(β chloroethyl)amine

lymphosarcomas during the course of investigations seeking means of combating these chemical warfare agents. It is interesting to note at least one earlier test of sulfur mustard against a mammary cancer in man (Adair and Bagg, 1931). The dramatic results obtained by the Yale group of investigators in 1942 (Goodman *et al.*, 1946) led to the organization of a program to arrange for, and to control, tests of methyl bis(β-chloroethyl)-amine (HN_2) against cancer. This was carried out largely within the chemical warfare research projects under the sponsorship of the Chemical Warfare Service and the Office of Scientific Research and Development. The National Research Council Committee on Atypical Growth served in an advisory capacity. Within four years a statement (Rhoads, 1946) could be made for the committee: The following summarizes its conclusions on the nitrogen mustards for use in the treatment of neoplastic diseases:

"(a) They are not a cure for such neoplastic diseases as have been studied.

"(b) The nitrogen mustards in large enough doses are injurious to many types of tissue; they appear to exert their greatest effects on rapidly growing tissue, presumably either normal or neoplastic.

"(c) Their predominant toxicologic effect is damage to normal hemopoietic function. The extent of this injury is the limiting factor in determining the amount that can be given to an individual. In some cases the hemopoietic injury exceeds the effect on the tumor.

"(d) The tumor regressions induced by these compounds (even with maximum dosages) are temporary, with maximal persistence rarely extending beyond several months.

"(e) The effects of the nitrogen mustards are in many respects similar to those of x-rays. It should be noted, however, that the great advantage of radiation therapy is that it can be given locally."

The studies supporting this statement were published shortly thereafter (Goodman *et al.*, 1946; Jacobson *et al.*, 1946; Karnofsky *et al.*, 1947b) Other reports, including recent reviews, appear to bear out the essential correctness of the initial summary (Boyland *et al.*, 1948; Gellhorn and Jones, 1949; Karnofsky, 1950; Karnofsky and Burchenal, 1950).

The clinical usefulness of the nitrogen mustards dictated its study in experimental animals. These agents offered a challenge to any cancer chemotherapy screening program to determine whether it could detect the activity of the mustards and, if so, to find more satisfactory derivatives of greater effectiveness or lowered toxicity. Also, in spite of the extensive war-sponsored studies there remained much to learn about the mode of action of the nitrogen mustards. It has been apparent that the mustards attack rapidly proliferating tissue (Philips, 1950).

Bass and Freeman (1946) reported bis(β-chloroethyl) sulfide caused regression or inhibited the growth of the P1534 and 6C3HED mouse lymphosarcomas. No change was noted with myeloid leukemia 1498, mammary carcinoma dbr B, spindle-cell carcinoma 15091a, or malignant melanoma S91. Many nitrogen mustards have been tested against mouse leukemia (Burchenal *et al.*, 1948a) and mouse tumors (Shapiro *et al.*, 1949). Some of the more effective mustards in the mouse trials have been tested in man (Burchenal *et al.*, 1948b, 1949a). Haddow *et al.* (1948a) have examined various aromatic β-chloroethyl amines for their action against the Walker rat carcinoma 256 and found β-naphthyl bis(β-chloroethyl)-amine the most effective. Extensive clinical trials of this mustard against Hodgkins disease have produced benefits without nausea and vomiting attending its use (Boyland, 1952). Of numerous nitrogen mustards tested against sarcoma 180, 3-bis(β-chloroethyl)aminomethyl-4-methoxymethyl-5-hydroxy-6-methyl pyridine dihydrochloride appeared best and was found in comparative studies superior to HN_2 (Stock *et al.*, 1951a). This was true also for certain other tumors (Sugiura and Stock, 1952). It was tested clinically and, while it was beneficial and can be given orally, there are drawbacks to its use. Its complete clinical evaluation was prevented by the finding of TEM to be a superior agent (Karnofsky *et al.*, 1951).

The similarity of the effects produced by the nitrogen mustards to those from x-rays has been pointed out frequently (Graef *et al.*, 1948; Boyland, 1948, 1952; Philips, 1950). Although many of the ultimate effects of the two agents are alike, those who have been unhappy in use of the term radiomimetic, coined by Dustin (1947), can take comfort that differences in effects of the two agents have been observed in careful cyto-

logical studies by Koller and Casarini (1952), also (Friedenwald *et al.*, 1948). One of the effects from both agents is the production of tumors in mice (Boyland and Horning, 1949). Heston (1949, 1950) also has noted the production of pulmonary tumors in mice. The carcinogenic and chemotherapeutic properties of the nitrogen mustards have provided added support for the concept that cancer chemotherapeutic agents are themselves likely to be carcinogenic (Haddow, 1949).

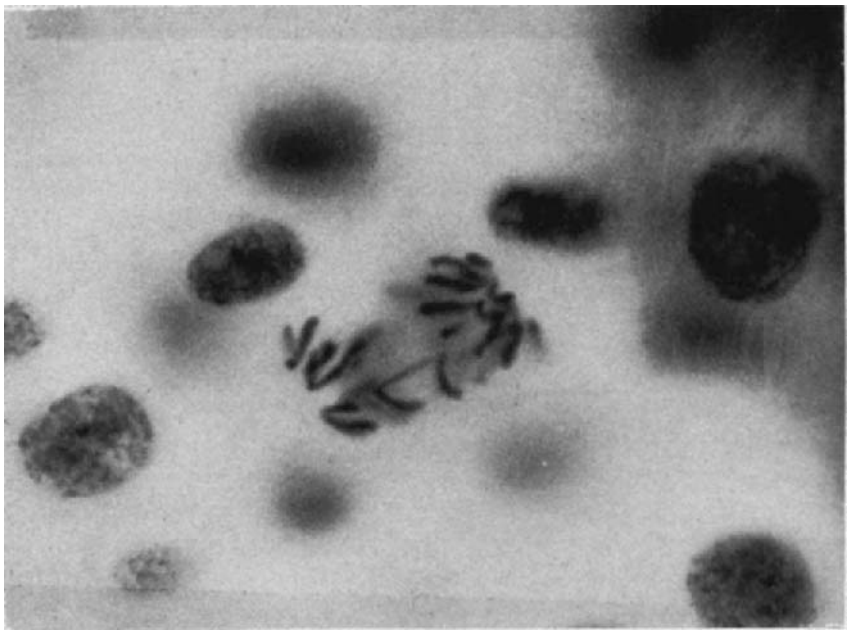


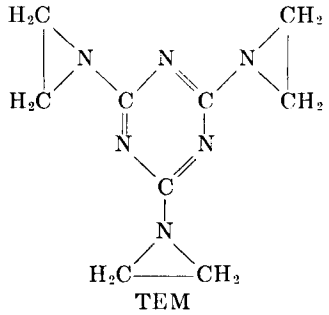
FIG. 4. Onion root tip mitotic figure in Feulgen-squash preparation 92 hours after 30 minutes exposure to HN_2 1 mM./liter. ×1350. Note dicentric chromosome forming bridge. (Courtesy of John J. Biesele.)

The consistent observation that the most effective nitrogen mustards possess two reactive groups (Karnofsky *et al.*, 1947a; Burchenal *et al.*, 1948a; Boyland, 1952; Loveless, 1951) led Goldacre *et al.* (1949) to suggest that the mustards acted by virtue of their ability to form links between chromosomes. Such linkages would interfere with the separation of the chromosomes during mitosis. This would result in bridges and breaks (Fig. 4.). Evidence that this attractive hypothesis is not an essential explanation was soon found in the form of compounds with only one reactive group which, however, possess antitumor activity as well as the ability to cause the chromosome abnormalities (Biesele *et al.*, 1950; Loveless and Ross, 1950). The monofunctional compounds are considerably less effec-

tive against tumors than the bis or polyfunctional compounds. Alexander *et al.* (1952) found in tests of "radiomimetic" compounds in wool and serum albumin that there is no correlation between the biological activity of a compound and its effectiveness as a crosslinking agent in the systems examined. The crosslinking hypothesis served to stimulate the investigators at the Chester Beatty Institute to produce a number of compounds to test their hypothesis (Ross, 1953). Among those prepared have been ethylenimines, epoxides (Everett and Kon, 1950), sulfonic acid esters (Timmis, 1951), and bis halogenoacetylbenzenes (Ross, 1950a). In addition to tests of these compounds against tumors, they have been studied for their chemical reactivities (Ross, 1950b,c) and particularly their physical chemical effects in certain model systems to provide more information upon their possible modes of action against tumors. Included in the studies have been demonstrations of similarities in the action of ionizing radiation and of the mustards on deoxyribonucleic acid (Butler *et al.*, 1950; Butler and Smith, 1950; Conway *et al.*, 1950). Alexander (1952) studied the interaction of various "radiomimetic" compounds with thymus nucleic acid and found that monofunctional compounds were not as effective as polyfunctional compounds in reducing the affinity of the nucleic acid for protamine. He offered the hypothesis that the initial effect of x-rays or the mustards on thymus nucleic acid might not be degradative but due to changes in the physical properties of the macromolecules. This idea was tested by Alexander and Fox (1952) with polymethacrylic acid as a model system. The data indicated a degradation from the action of x-rays whereas the nitrogen mustard appeared to change the shape of the large molecule. The authors were tempted to suggest similar effects on nucleic acid from these agents. These studies, those of Koller and Casarini, previously referred to, and those of Loveless and Revell (1950) indicating action of the agents at different stages in cell development, point to definite differences in the chemical mechanism of the action of mustards and x-rays. Therefore, the mimicry of x-rays by the "radiomimetic" substances appears to pertain more to the end results rather than the fundamental mechanism of action. Thus the concept of radiomimetic substances (Dustin, 1947), though shown not to be completely applicable, has served the purpose of stimulating research just as has another concept, that of crosslinking (Goldacre *et al.*, 1949), which also has been found not essential as an explanation for the action of the β-chloroethylamines and other reactive substances.

(b) *Ethylenimines:* triethylenemelamine, (2,4,6-tris(ethylenimino)-s-triazine) (TEM). It was natural that investigators thinking of crosslinking agents or of chemically reactive compounds capable of polymerization should have considered the triazine TEM, which had been described

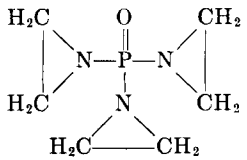
as a crosslinking agent for wool. It has been assumed (Philips, 1950) that HN_2 acts through formation of an ethylenimonium ring and thus it was also natural that ethylenimines should have been tested on the basis of that structural relationship. TEM was studied independently in a number



TEM

of laboratories. Lewis and Crossley (1950) in experiments giving the compound in the diet and Burchenal *et al.* (1950b) reported action against mouse sarcoma and leukemia, respectively. Sarcoma 180 was shown to be markedly inhibited by TEM (Stock and Buckley, 1950; Buckley *et al.*, 1950, 1952). This was confirmed by Goldberg and Schoenbach (1951). Reports of independent studies revealed the effectiveness of TEM against the Walker carcinoma 256 (Hendry *et al.*, 1950; Haddow, unpublished). TEM was shown to have a marked effect against other solid tumors (Table I) (Sugiura and Stock, 1952; Crossley *et al.*, 1951b) and the Ehrlich ascites tumor (Sugiura and Stock, 1951). It appears to be effective against those tumors responding to the nitrogen mustards. That this is true for human cancer was discovered early (Rhoads *et al.*, 1950; Karnofsky *et al.*, 1951) and amply confirmed (Wright *et al.*, 1950, 1952b; Paterson and Boland, 1951; Boyland, 1952; Bayrd *et al.*, 1952). Also confirmed was the fact that TEM could be given orally with little or no nausea and vomiting. The study of the pharmacology of TEM reported by Philips and Thiersch (1950), emphasized similarities to nitrogen mustards in its toxicological action.

Other ethylenimines. Various ethylenimines more or less closely related to TEM have been tested. Included were the less effective ethyleneureas (Buckley *et al.*, 1952; Hendry *et al.*, 1951c). Ethylenimino pyrimidines



N,N′,N″-Triethylenephosphoramide

would appear to be of definite value (Elion *et al.*, 1952; Hendry *et al.*, 1951c) and possible prospects for clinical trial. An extension of the studies on ethylenimines produced the ethylenephosphoramides. Buckley *et al.* (1951b) reported anti-sarcoma 180 activity from the phosphoramides (Fig. 5) in which there appears to be no greater degree of effect than from TEM, but possibly there is a somewhat greater therapeutic dose range. Burchenal *et al.* (1952a) reported benefits in mouse leukemia with phosphoramides. Crossley *et al.* (1951a,b) reported on the effectiveness of

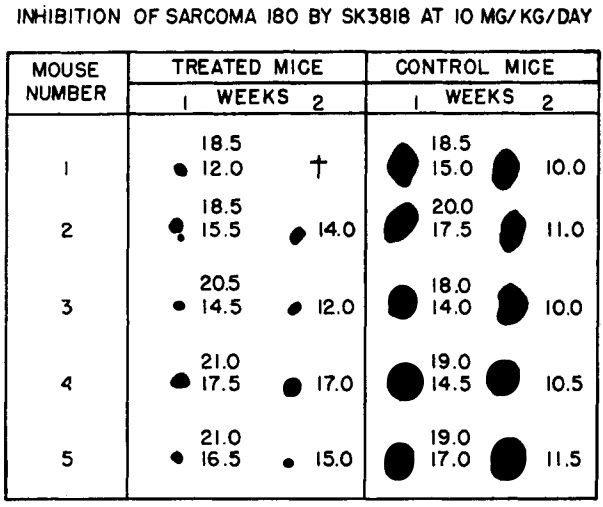
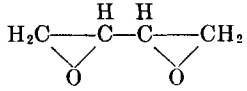


FIG. 5. Area diagram of tumors in control mice and in those treated for one week with N, N′, N″ triethylenephosphoramide at a dose of 10 mg./k./day. The intraperitoneal injections were terminated at the time of the first week measurement. (Reprinted with permission from *Proc. Soc. Exptl. Biol. Med.* **78**, 300, 1951.)

several phosphoramides, N,N′,N″-triethylenephosphoramide and N,N-diethyl-N′,N″-diethylenephosphoramide, and also on the tetramethylene, the pentamethylene, and the (3-oxapentamethylene) substituted phosphoramides against rat sarcoma (Crossley *et al.*, 1952a,b). They have advocated using intermediate dose levels in order to obtain better results presumably by allowing the host's defences to play a role. In tests of these and other phosphoramides Williams *et al.* (1952) on the basis of stability and optimum treatment doses selected as worthy of clinical trial these additional ethylenimines: N-pentamethylene-N′,N″-diethylenephosphoramide, N-(acrylamido-methyl)-3-ethyleneiminopropionamide, and N,N′,N″-tris(2-methylethylene)phosphoramide. Preliminary clinical observations on the phosphoramides, N,N′,N″-triethylenephosphoramide and N,N-diethyl-N′,N″-diethylenephosphoramide (Farber *et al.*, 1953;

Sykes *et al.*, 1953) have not revealed as yet any advantage over TEM. Recently N,N′,N″-triethylenethiophosphoramide has been reported the most active in preventing metastases of rat mammary adenocarcinoma R2426 (Personeus *et al.*, 1952).

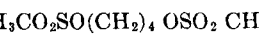
(c) *Epoxides.* Hendry *et al.* (1951b) in an extension of studies started



Butadiene dioxide

with methylolamines (Hendry *et al.*, 1951a) found that the bis-epoxides, such as butadiene dioxide, were inhibitory for the Walker rat carcinoma 256 but only at toxic levels. In addition, the compounds were found to be carcinogenic; therefore, it was concluded that they would be unlikely to have any therapeutic application. The epoxides failed to inhibit sarcoma 180, which has appeared to be somewhat less sensitive than several rat tumors to the mustards and related compounds (Stock *et al.*, 1950).

(d) *Esters of sulfonic acids.* Another group of compounds with alky-

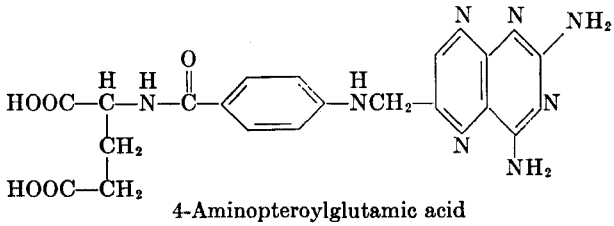


1,4-Dimethanesulfonoxybutane

lating capacity are those in a series of which 1,4-dimethanesulfonoxybutane is representative. Activity of the compounds has been determined by cytological observations (Koller, unpublished). A number of them have been tested to determine the influence of variations in structure upon cytotoxicity. Based upon these results Timmis (1951) has postulated that the compounds are effective as a result of their attachment at each reactive end of the molecule to some atom, e.g., amino N, in a biologically important molecule. Lending support for this concept are the facts that the sulfonic acid esters incapable of forming such rings are ineffective whereas the most active ester is the one that theoretically should form the most stable ring. These compounds have been found active against the Walker carcinoma 256 (Haddow and Timmis, 1950, 1953) and sarcoma 180 (Clarke, unpublished) and other solid tumors (Sugiura, 1953). Galton and Timmis (1952) and Galton (1953) have reported preliminary clinical data indicating effectiveness of 1,4-dimethanesulfonoxybutane in chronic myelogenous leukemia. Among the first nineteen cases remissions have been produced, which in the best cases were for 6 to 23 months.

B. *Antimetabolites.* (a) *Folic acid analogs.* Discovery of the antitumor activity of the 4-amino folic acid analogs arose from studies actually pointing in the opposite direction. This situation has been reviewed in detail by Rhoads (1949) and by Gellhorn and Jones (1949). Lewisohn and his asso-

ciates studied the influence of extracts of a number of natural materials upon various mouse tumors. Beef spleen extract was reported to cause regressions of sarcoma 180 (Lewisohn, 1938). Next the spleens of mice, "healed" of their tumors, were used for preparation of extracts cytotoxic for spontaneous mammary adenocarcinoma in mice. As this could hardly be considered a practical source of material, these investigators turned their attention to screening extracts from a number of other natural sources. Extracts of barley and brewer's yeast were found to cause regressions of mammary adenocarcinoma (Lewisohn, 1947) and, as a consequence, attention was focused upon the vitamin B complex. Inositol was



4-Aminopteroylglutamic acid

considered the only one of the crystalline B vitamins in yeast active against the mouse tumors (Lazlo and Leuchtenberger, 1943a). Folic acid was reported as capable of causing complete regression of breast cancer in mice (Leuchtenberger *et al.*, 1944, 1945) but later the factor was identified as the *Lactobacillus casei* factor, pteroyltriglutamic acid (Lewisohn *et al.*, 1946). Hesselbach *et al.* (1947) and Morris (1947) were unable to obtain results consistent with studies of the Lewisohn group. Sugiura (1947) in an attempt to duplicate precisely the experiments was unable to confirm the results of Lewisohn *et al.*

Farber *et al.* (1947) after reviewing the data from Lewisohn's laboratory, tried pteroyldi- and triglutamic acids in 90 patients. Some cases of diminished pain, improved energy and appetite, and a sense of well-being, all without any indication of toxicity, were reported observations. It was at a meeting in which clinical findings similar to the above were reported that there was presented the first evidence that tumors were affected by the 4-amino folic acid analogs (Woll, 1948; Little *et al.*, 1948a,b,c). Clinical evidence of the value of the 4-amino folic acids was first presented by Farber *et al.* (1948) who demonstrated temporary remissions were induced in acute leukemia of children by aminopterin (4-aminopteroylglutamic acid). In this communication acceleration of the leukemic process was reported seen in children with acute leukemia after administration of pteroyldi- or triglutamic acids. Farber's clinical observation on the effectiveness of the 4-amino folic acids has been confirmed although with some differences in degrees of success (Dameshek, 1949; Dameshek *et al.*, 1950; Burchenal

et al., 1951b; Berman *et al.*, 1949; Meyer *et al.*, 1949; Wright *et al.*, 1951; Sawitsky *et al.*, 1952; Schoenbach *et al.*, 1952).

Animal experimentation was quick to show that the 4-amino folic acid analogs were potent inhibitors of the development of the leukemic process in mice (Burchenal *et al.*, 1949b; Law *et al.*, 1949) and also of the growth of

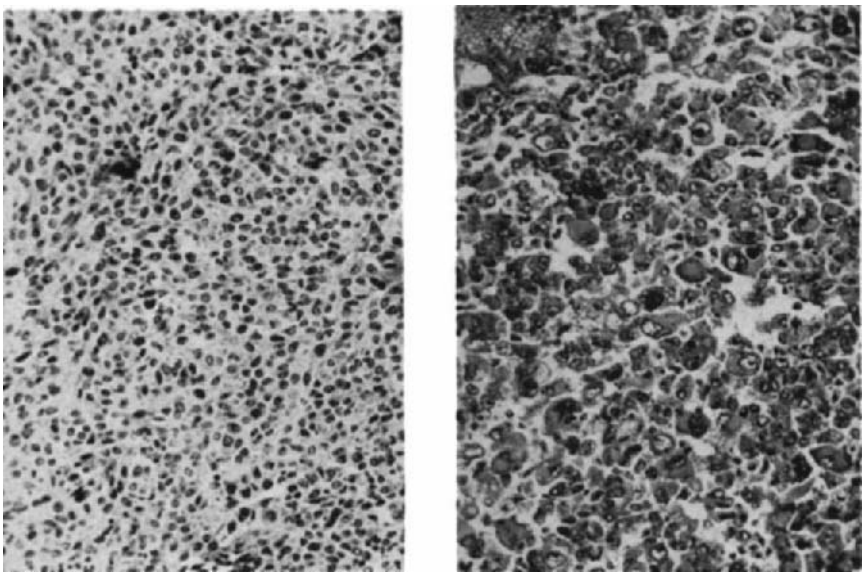


FIG. 6. (Left) Picture of an untreated rat sarcoma R39. Note the regular size of tumor cells and large nuclei. (Hematoxylin and eosin stain. ×180.) (Right) Rat sarcoma R39 after treatment with aminopterin (daily doses of 0.25 mg./kg. on six consecutive days). Tumor cells generally enlarged with large vesicular nuclei, cytoplasm granular. Note degenerating nuclei and tumor debris and also a number of viable tumor cells. ×180. (Courtesy of *Cancer*.)

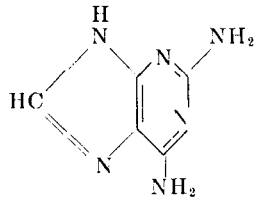
sarcoma 180 in mice (Moore *et al.*, 1949; Schoenbach *et al.*, 1949; Stock *et al.*, 1950) and other tumors (Higgins and Woods, 1949; Sugiura *et al.*, 1949). Figure 6 illustrates the type of cytological damage observed in the tumors of animals given the 4-amino folic acid analogs. The animal tests also revealed the lack of activity of folic acid and its glutamic acid conjugates against some of the above tumors. In tissue culture the 4-amino folic acids appear to have little or no toxicity for tumor cells (Stock *et al.*, 1950; Plummer, 1952). The findings with folic acid analogs in experimental animals of antineoplasia activity as a counterpart of observations in man provided added confidence in the ability of the animal test procedures to detect compounds of potential clinical usefulness.

Further animal studies with the folic acid analogs and related com-

pounds have been conducted to learn something of their limitations and of their mode of action. Philips and Thiersch (1949b) have described the toxic manifestations of aminopterin in mice and rats following acute and chronic administration. These studies, considered essential prior to clinical trial, revealed the symptoms of weight loss, hypoplasia of bone marrow, and intestinal lesions with diarrhea. Death quickly followed appearance of the syndrome. Ferguson *et al.* (1950) reported similar findings in mice, rats, and dogs caused by administration of amethopterin (4-amino-N^{10}-methylpteroylglutamic acid). Thiersch and Philips (1949) in their study of aminopterin on dogs called particular attention to observations of megaloblastosis. These studies served to bring out limitations in the usefulness of the 4-amino folic acids which are to be found in a susceptibility of the most sensitive normal cells to damage from these drugs equal to that of the neoplastic cells. The parallel in susceptibility to, and recovery from, the action of amethopterin was shown for bone marrow and sarcoma 180 in mice (Thiersch and Stock, 1949). Burchenal *et al.* (1949d) tested 90 compounds related to folic acid against mouse leukemia. Included were purines, pyrimidines, pteridines, and several other types of nitrogen heterocylics. Of this group only four compounds, aminopterin, amethopterin, 4-amino-9-methylpteroylglutamic acid and 4-amino-9,10-dimethylpteroylglutamic acid were found effective. Skipper *et al.* (1950a) in tests of certain compounds related to the pteridine and other moieties of folic acid found none of the 14 compounds studied was active in increasing the survival time of leukemic mice. A number of investigators have sought to block the action of the 4-amino analogs against tumors. In that respect folic acid has not been as effective as citrovorum factor (Burchenal *et al.*, 1950a; Goldin *et al.*, 1949a). These data add weight to the belief that the 4-amino folic acid analogs function by interference with the conversion of folic acid to citrovorum factor (Welch and Heinle, 1951). Many of the investigations of the folic acid antagonists which have not been conducted in connection with problems of neoplasia but which have provided much useful information are summarized in several reviews (Jukes and Stokstad, 1948; Petering, 1952).

(b) *Purines: 2,6-diaminopurine.* The first demonstrated biological activity for this interesting compound was as an antagonist, within defined ranges of concentration, for pteroylglutamic acid and for adenine in the metabolism of *Lactobacillus casei* (Hitchings *et al.*, 1948). Shortly, thereafter, in studies based on the bacteriological data, this purine was found to possess the ability to prolong the lives of AKR mice with transplanted leukemia (Burchenal *et al.*, 1949c). This was confirmed by Skipper *et al.*, (1950b). Effects of 2,6-diaminopurine and their blockage by adenine in numerous biological systems (Table II) suggest as a mechanism of action

interference with purine metabolism by diaminopurine. Test of this purine against various solid tumors in mice and rats indicates in general a lack of antitumor activity at tolerated doses (Table I) (Stock *et al.*, 1950); however, in tissue culture, as mentioned previously, the differential action of



2,6-Diaminopurine

2,6-diaminopurine has been beautifully demonstrated. The negligible *in vivo* activity in experimental tumors appears to be reflected in the clinical trials conducted thus far. In a limited group of patients only the occasional patient appears to have been benefited (Burchenal *et al.*, 1951b). A number of thiazolino analogs of purines related to 2,6-diaminopurine have been

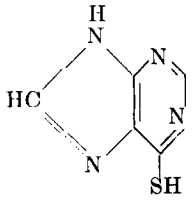
TABLE II
Biological Activities of 2,6-Diaminopurine

Activity	Reference
L. casei growth inhibition in defined medium	Hitchings *et al.*, 1948
Inhibition growth of estrogen stimulated chick oviduct	Hertz and Tullner, 1949
Interference in vaccinia virus production in tissue culture	Thompson *et al.*, 1949
Benefits in mouse leukemia	Burchenal *et al.*, 1949
Bone marrow hypoplasia, in rats and dogs	Philips and Thiersch, 1949a
Production of anemia in chick embryo	Karnofsky *et al.*, 1949
Bone marrow hypoplasia, etc., in swine	Cartwright *et al.*, 1950
Chromosome abnormalities in mouse	Dustin, 1950
Differential toxicity to normal and cancer cells of rat and mouse	Biesele *et al.*, 1951
Interference with Russian encephalitis virus production in tissue culture	Friend, 1951
Limited mouse protection from Russian encephalitis virus	Moore and Friend, 1951
Chromosome abnormalities in tissue culture	Biesele *et al.*, 1952
Decrease in kappa content of *Paramecia aurelia*	Stock *et al.*, 1952

found without effect in tests against myeloid leukemia C1498 and mammary adenocarcinoma EO771 (Gordon *et al.*, 1951).

Other purines. 6-Mercaptopurine, 6-chloropurine, and unsubstituted purine recently have shown an ability to inhibit sarcoma 180. Although

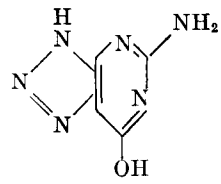
the inhibition has been only moderate (Clarke *et al.*, 1953a,b) 6-mercaptopurine, the first of the three found to have activity, attracted attention because it appeared to produce a more lasting damage to the tumor than observed with other tumor inhibitory substances. Preliminary clinical trials in acute leukemia indicate that interest in the compound is justified (Burchenal *et al.*, 1953a,b).



6-Mercaptopurine

Parsons *et al.* (1947) reported a slight inhibition of mouse sarcoma grafts by yeast adenylic and guanylic acids while cytidylic acid had no effect and uridylic acid showed a growth-promoting effect. Barker *et al.* (1949) tested a few purines and pyrimidines against several mouse tumors. 4-Aminouracil showed a slight inhibition. Ambrus *et al.* (1951) have reported adenosine-3-phosphoric acid inhibits while the 5-analog enhances growth of the subcutaneous Ehrlich carcinoma. Adenosine triphosphate showed no effect.

(c) *Pyrimidines: 8-azaguanine:* 5-amino-7-hydroxy-1H-v-triazolo (d) pyrimidine, (guanazolo). This compound might well be considered with the purines for, while it is a substituted pyrimidine, it is of interest because of its biological activity as an antagonist of guanine. It was prepared for test of its antibacterial activity by Roblin *et al.* (1945). Kidder and

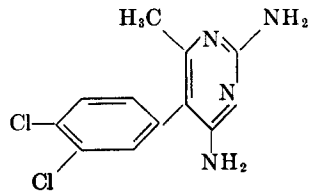


8-Azaguanine

Dewey (1949) in nutritional studies on *Tetrahymena* found this organism requires guanine and that 8-azaguanine acts as a potent antagonist in this biological system. The test of 8-azaguanine against tumors in mice (Kidder *et al.*, 1949) was made based upon the assumption that the nutritional requirements of the tumors would be similar to that of the tetrahymena, at least with respect to guanine metabolism. The first three tumors studied to test the hypothesis were inhibited to such a degree that it was con-

cluded all tumors might possess a purine metabolism distinct from that of normal cells. It was soon revealed that this generalization could not be supported by existing experimental data (Stock *et al.*, 1949a). Additional studies stimulated by the interesting initial report on tumor inhibition clearly indicated the antitumor activity of this compound is restricted chiefly to mammary adenocarcinomas and some leukemias (Gellhorn *et al.*, 1950; Sugiura *et al.*, 1950a; Law, 1950). Various studies have extended the information on 8-azaguanine in cancer (Kidder *et al.*, 1951; Meyer and Weinmann, 1951; Finkelstein and Thomas, 1951; Finkelstein *et al.*, 1951). The role of guanine in cancer has been considered by Graff *et al.* (1951). A number of the investigators referred to earlier have found guanine does not block the antitumor action of 8-azaguanine. Goldin *et al.* (1950), however, have reported a minimal reversal of the 8-azaguanine action and a greater effect from guanylic acid, which appeared limited in part, at least, by a growth inhibitory effect of the guanylic acid. 8-Azaguanine appears not to interfere with the incorporation of either guanine or 4-amino-5-imidazole carboxamide into tumor or liver (Carló and Mandel, 1953). Investigations of the ability of various tumors to convert 8-azaguanine to 8-azaxanthine by the action of guanase suggested a partial explanation for failure of 8-azaguanine to affect some tumors (Hirschberg *et al.*, 1952, 1953). Initial clinical studies (Armistead *et al.*, 1949) revealed that parenteral 8-azaguanine causes a severe dermatitis, nausea, vomiting, and diarrhea. In another investigation most of these side reactions were noted but eleven patients were studied with three of them (two of Hodgkin's disease and one lymphosarcoma) believed to show some temporary improvement (Wright *et al.*, 1952a). Colsky *et al.* (1952) administered 8-azaguanine orally, IM, and IV to 13 patients without significant benefit. The side effects were observed except when the oral dosage was used.

2,4-Diaminopyrimidines. Among numerous pyrimidines tested for antitumor activity have been the antimalarial, Daraprim, (2,4-diamino-5-(*p*-chlorophenyl)-6-ethyl pyrimidine) and related compounds (Falco *et*



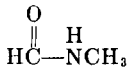
2,4-Diamino-5-(3′,4′-dichlorophenyl)-6-methyl pyrimidine

al., 1951). Although Daraprim has not been demonstrated to have antitumor activity, a number of related pyrimidines have been found mildly in-

hibitory for mouse sarcoma 180 (Clarke *et al.*, 1952). The effective pyrimidines have been substituted with amino groups in both the 2 and 4 positions. In the 5 position has been a bis substituted (usually 3′,4′-dichloro) phenyl group. The corresponding benzyl and phenoxy substitutions have yielded negative compounds. The 6 position of the active pyrimidines has held H, methyl, ethyl and *n*-amyl groups. Consistent with observations on the antifolic acid activity of these compounds in bacteria, the toxicity and antitumor effects of these pyrimidines can be nullified by citrovorum factor and partially by folic acid. Burchenal *et al.* (1952b) reported several of the diaminopyrimidines effective in prolonging the lives of mice with AK4 leukemia and capable of causing a drop in white cell count in mice with either the amethopterin sensitive or resistant leukemias. It is of some theoretical interest that a compound similar to the tumor inhibitory diaminopyrimidines, 2,4-diamino-5(*p*-chlorphenyl)-6,6-dimethyl-5,6-dihydro-1,3,5-triazine has been reported to cause cytologically determinable damage to sarcoma 180 (Farber *et al.*, 1952, 1953b). The ability of the triazine to inhibit the growth of sarcoma 180 appears to be lacking under conditions in which the diaminopyrimidines are moderately active. 2,4-Diamino-5(3′,4′-dichlorphenyl)-6-methyl pyrimidine recently has gone to clinical trial but has proved more difficult to handle because of problems in toxicity greater even than those of the 4-amino folic acid analogs (Murphy, unpublished).

A few pyrimidines tested against mouse tumors by Barker *et al.* (1951) showed a slight inhibitory effect of 2,4,6-triaminopyrimidine and of 2-hydroxy-4,6-diaminopyrimidine.

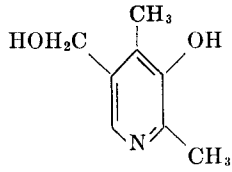
(d) *Formamides.* Formamide at rather toxic doses and N-methyl formamide at tolerated dose levels are capable of retarding moderately the growth of sarcoma 180 (Clarke *et al.*, 1953c). Among approximately 60



N-Methyl formamide

substituted formamides tested, only two have been found active. It is particularly interesting that such simple, closely related compounds as acetamide, thioformamide, dimethyl formamide, and N-ethyl formamide have failed to show the ability of formamide and N-methyl formamide to influence the growth of sarcoma 180. As this is being written it is uncertain in which category the formamides should be placed. It is possible that they may be considered as antagonists in the metabolism of nucleic acid precursors through interference with the incorporation of formate into purines. The suggestion also has been made that the action of urethane and the formamides may be interrelated (Skipper, unpublished).

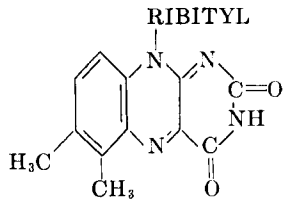
(e) *Pyridoxine analogs: Deoxypyridoxine.* Stoerk (1947) showed that established 6C3HED lymphosarcoma implants regressed markedly following administration of a pyridoxine antagonist, deoxypyridoxine, coupled with a pyridoxine deficient diet. Loefer (1951) with a rat fibrosarcoma



Deoxypyridoxine

showing a low per cent of takes noticed no significant difference in the takes of controls and deoxypyridoxine treated rats. A borderline inhibition has been noted with deoxypyridoxine but not with methoxypyridoxine in the retardation of the growth of sarcoma 180. Possibly the difference may be explained by the observation that methoxypyridoxine is not an antagonist in the mouse and may actually provide pyridoxine (DeRengo and Cerecedo, 1950). Several compounds containing deoxypyridoxine and methoxypyridoxine moieties with β-chlorethyl groups presumably are effective as nitrogen mustards (Stock *et al.*, 1951a).

(f) *Riboflavin analogs.* A diet low in riboflavin produced regression of



Isoriboflavin

well established 6C3HED lymphosarcomas in C3H mice (Stoerk and Emerson, 1949). The regression was enhanced by simultaneous feeding of the riboflavin antagonists, isoriboflavin, and galactoflavin. Survival was prolonged in most instances. In similar experiments other vitamin antagonists, pyrithiamine, 3-acetylpyridine, and pteroylaspartic acid, had no influence. Reference will be made below to the combinations of steroid and riboflavin antagonists. The diethyl analog of riboflavin has been reported to cause a slight effect upon the Walker rat carcinoma 256 (Aposhian and Lambooy, 1951). Holly *et al.* (1950) found in tests of various 6,7-dichloro-9-(1′-R)-isoalloxazines that only the compound in which R is d-sorbityl was there caused any regression of established 6C3HED lymphosarcomas.

(g) *Other vitamin antagonists.* To test the hypothesis that a biotin deficiency should be harmful to cancer Rhoads and Abels (1943) fed a few

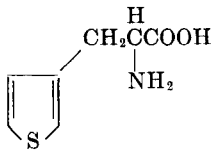
patients raw egg white to utilize its avidin content to bind biotin. No benefits nor signs of biotin deficiency were observed.

Montanez *et al.* (1951) have looked for an influence of pantothenic acid deficiency induced by deficient diet and an analog upon a rat fibrosarcoma. The observed retardation in growth appeared to reflect the total systemic effect upon the host. Morris and Lippincott (1941) previously had reported that dietary pantothenic acid deficiency retarded the growth of spontaneous mammary adenocarcinomas in C3H mice. Bischoff *et al.* (1943) failed to find that such a diet would retard sarcoma 180 although it was recognized that there may not have been depletion of this vitamin.

The effectiveness of the 4-amino folic acid analogs naturally has directed the attention of many chemotherapists to antagonists of vitamin B_{12}. As a result these have been impatiently awaited since the announcement of the crystallization of vitamin B_{12} (Rickes *et al.*, 1948). A suggestion of the possible value of B_{12} antagonists may be read into the data of Oleson and Little (1949) demonstrating that vitamin B_{12} has a role in the growth of Rous sarcoma comparable to that previously shown for folic acid. Furthermore Woolley (1952, 1953) believes he has evidence that certain tumors have the ability to synthesize vitamin B_{12}. On this basis he has suggested the desirability of studying B_{12} antagonists for antitumor activity.

(h) *Amino acid analogs.* Various investigators have considered the desirability of using modified amino acids for the chemotherapy of cancer. Fluoro and sulfonic acid analogs and N-iodoacetylated amino acids have yielded little thus far (Greenberg and Schulman, 1947; Friedman and Rutenburg, 1950; Niedner, 1941; Stock *et al.*, unpublished). Some basis for such studies may be found in experiments reviewed by Tannenbaum and Silverstone (1949) revealing detrimental influences upon tumor growth as a result of protein and amino acid deficiencies.

Jacquez *et al.* (1951, 1952) have observed in tissue culture that β-2-thienylalanine acts as a phenylalanine antagonist and that it is more toxic for mouse sarcoma cells than normal cells. It was further noted that tumor cells of various types usually lacked a property generally possessed by the normal tissues included in the study, namely the ability to utilize β-2-thienylalanine in transaminations.



β-2-Thienylalanine

C. *Steroids.* The earliest investigations of the relationship between endocrine influences upon tumors and the most extensive ones subsequently have been concerned with the regulation of tumor growth by endocrine glands and steroids of the sex hormone group which they produce. Well-known studies in this area are those of (Civiale, see White, 1893; Lathrop and Loeb, 1916; Loeb, 1919; Gardner, 1947, 1953; Loeser, 1938; Huggins, 1942, 1946; Nathanson, 1947; Adair *et al.*, 1949; Laccasagne, 1939; Lipschutz, 1950). The clinical studies of the steroids and the background have already been well reviewed (Gellhorn and Jones, 1949). The latest development pertaining to this area has been the use of adrenalectomies for human mammary and prostatic cancers (Huggins and Bergenstal, 1952). Additional time will be required for an accurate evaluation of the benefits initially reported.

Numerous reports on the influence of steroids other than the sex hormones have appeared particularly since the demonstration by Heilman and Kendall (1944) of the ability of cortisone (compound E) to inhibit the growth and cause regression of a mouse lymphosarcoma. Earlier Sugiura (1931) had found no effect of an extract of suprarenal cortex on a number of mouse and rat tumors; however, these later were found unsusceptible also to cortisone. Dobrovolskaia-Zavadskaia and Zephiroff (1939) reported adrenal cortex extracts tended to diminish the rate of growth of tumors and prolong life of the hosts. Murphy and Sturm (1944), Law and Spiers (1947), Diller *et al.* (1948) have found that adrenal cortical extracts could inhibit the development of transmitted leukemia in rats and mice and cause some regression of mouse sarcoma 37. These results anticipated the finding of dramatic but temporary regressions in human lymphoid tumors and acute leukemia caused by ACTH and cortisone (Pearson *et al.*, 1949).

When adequate supplies of cortisone permitted, the observations of Heilman and Kendall were quickly confirmed and extended. Sugiura *et al.* (1950b) reported the inhibition of mouse lymphosarcomas by compounds E, F, and A. Emerson *et al.* (1950) also noted detrimental effects of cortisone upon mouse lymphosarcoma 6C3HED which were accentuated by a riboflavin deficiency in the host. Woolley (1950a,b) reported an increase of life span of those mice with lymphatic leukemia P1534 which were administered compound A. Higgins *et al.* (1950a) observed an inhibitory effect on a mouse rhabdomyosarcoma and a partial inhibition of a transplanted mouse ependymoma (Brzostowicz *et al.*, 1951). The latter group of investigators also reported, in agreement with Burchenal *et al.* (1950c), some restriction of AK4 leukemic process in AKR mice although survival time was not appreciably increased. Woolley and Peters (1953) have shown that prolonged administration of cortisone to

AKR mice prevents their death from spontaneous leukemia at the usual time.

The stimulus to research from cortisone developments provided many additional steroids for study. In one program all steroids available in adequate amounts were tested in a spectrum of mouse tumors. In cases of limited quantities the restricted testing always included at least the

TABLE III
Relative Abilities of Selected Steroids to Inhibit the Growth of Mouse Lymphosarcoma[a]

Steroid	Dose, mg./k./day	Inhibition of Growth[b]
11-Dehydro-17-hydroxycorticosterone acetate (cortisone acetate) (compound E)	37.5	+
11-Dehydro-17-hydroxycorticosterone acetate (cortisone acetate) (compound E)	12.5	±
17-Hydroxycorticosterone acetate (Kendall's compound F acetate)	37.5	+
17-Hydroxycorticosterone acetate (Kendall's compound F acetate)	12.5	±
11-Dehydrocorticosterone acetate (Kendall's compound A acetate)	75	+
11-Dehydrocorticosterone acetate (Kendall's compound A acetate)	37.5	±
Corticosterone (compound B)	375	+
Corticosterone (compound B)	150	±
11-Deoxycortisone acetate	450	−
11,17-Deoxycortisone acetate	375	−
21-Deoxycortisone	375	−
Dihydro- and tetrahydrocortisones	375	−

[a] (Stock *et al.*, 1951b)
[b] Inhibition evaluated as follows:
+ no growth to growth ¼ the diameter of the controls.
± growth from ¼ to ¾ the diameter of the controls.
− growth greater than ¾ the diameter of the controls.

lymphosarcomas. Steroids available in quantities inadequate for mouse studies were tested for ability to inhibit the growth and development of the chick embryo. Of over 50 steroids tested against the lymphosarcomas only compounds E, F, A, and B were found inhibitory. Compounds E and F are equally effective while compounds A and B are considerably less inhibitory. See Table III. All the other steroids were without demonstrable activity against the Patterson and Mecca lymphosarcomas even when tested at thirty times the minimum effective dose of cortisone (Stock *et al.*, 1951b). These results allowed tentative conclusions on the relationship of structure to the antilymphosarcoma action (Stock,

1951). The formula of cortisone in Fig. 7 will assist in the following comments. The 11-oxygen function, whether keto or hydroxy group, is essential, as apparently is the 3-keto group with the Δ^4-conjugated unsaturation. The 21-hydroxy group appears essential, however, in other experiments 21-deoxycortisone was reported to be very weakly active (Woolley, 1950c; Emerson, 1951). While a 20-keto group has been present in the four active steroids, compounds to test the necessity of that group have not been available. Other recent reports of tests of cortisone against tumors include (Ingle *et al.*, 1950) and Ingle and Nezamis (1951), who

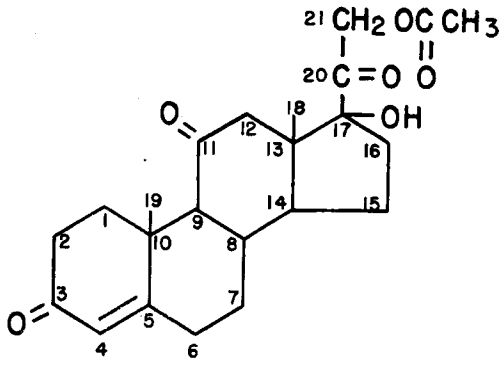


FIG. 7. Formula of cortisone (compound E) with numbered atoms for consideration of the relationship of structure to activity against mouse lymphosarcoma.

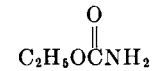
reported cortisone moderately inhibits the Walker carcinoma 256 and the Murphy rat lymphosarcoma. Foley and Silverstein (1951) made the interesting observation that in certain conditions cortisone may cause tumors to develop more extensively than controls. This foreshadowed the successful transplantations into alien mice of strain specific tumors (Foley, 1952) and of human tumors into rats (Toolan, 1953; Hoch-Ligeti and Hsü, 1953).

The best results with the steroids against the lymphosarcomas have been obtained at dose levels that could not be continued beyond a week of daily injections. The effects have been observed as temporary inhibitions of tumor growth. These findings appear to have their counterpart in the clinical studies (Pearson *et al.*, 1949).

The chick embryo has demonstrated its usefulness in the study of steroids (Karnofsky *et al.*, 1950). Cortisone was found to cause a characteristic stunting of the embryo. The picture seen is as follows: the embryo is small; the yolk sac and chorioallantoic membrane are not completely formed; the embryo is in a characteristic curved position with the amnion drawn tightly around it; it is pale and feathers are nearly absent. After the

action of cortisone was discovered, the chick embryo was studied with all other available steroids. It was found that compounds F, A, B, 21-deoxycortisone, and 11-ketoprogesterone were effective in smaller doses than cortisone. Nine other steroids in higher doses also stunted the embryo (Stock *et al.*, 1951b). All of the most active steroids contain an 11-oxygen group. It is not known whether all the effective steroids act in a similar manner against the embryo. It is also possible that the less effective steroids may be active by virtue of changes made in their structure by the chick embryo. As the technique employing the chick embryo appears to detect corticosteroids (particularly steroids active against the mouse lymphosarcomas) it would appear worth considering this procedure in searches for such activity especially when the amounts of test material are limited. In addition, Landauers' (1947) observations suggest the possibility of detecting the activity even in crude extracts.

D. *Carbamates.* The carbamates might be used as a class of compounds to argue both for and against simple screening procedures utilizing normal cells as a preliminary method of selecting compounds for test against tumors. Lefevre's (1939) description of abnormal development of seedlings and their retarded growth when exposed to ethyl phenyl carbamate was extended by a systematic survey of the aryl carbamates and related substances as plant growth inhibitors (Templeton and Sexton, 1945). These studies were adequate to attract attention to this class of compounds as one worthy of study in neoplastic growth; however, if the data from the



Urethane

plant studies had been relied upon, urethane, would have been neglected because of its lack of activity in the initial screening. Fortunately, Haddow and Sexton (1946), included urethane among the carbamates tested against the Walker rat carcinoma 256 and a mouse spontaneous mammary adenocarcinoma. Urethane and phenyl urethane appeared equally effective in inhibiting the growth of the mammary tumor. In the Walker tumor there was found a profound change in histological structure, suggesting maturation of undifferentiated cells; however, Lushbaugh *et al.* (1948), in a study on wound healing in an attempt to elucidate the mode of action of urethane, concluded that it does not inhibit cellular proliferations by causing inanition or premature cellular maturation.

Studies on the antitumor activity of urethane have been extended revealing inhibition of several experimental forms of cancer (Yu, 1947; Murphy and Sturm, 1947; Engstrom *et al.*, 1947; Law, 1947; Weir and Heinle, 1947; Güthert, 1949; Landschutz and Muller-Dethard, 1949).

Most of these studies were concerned with leukemias and no doubt were stimulated as much by the first clinical report (Paterson *et al.*, 1946) as by the first report on animal tumors. The results in the Walker 256 carcinoma had encouraged test of urethane in hopeless cases. While no benefit was observed in the human carcinomas, it was noted that urethane caused a fall in the white cell count which then suggested the trial of it against human leukemia. It was observed that temporary remissions occurred in myelogenous leukemia with less satisfactory results in chronic lymphatic leukemia and no benefit in acute leukemia. These initial observations have been borne out in subsequent trials (see review Karnofsky and Burchenal, 1950).

The observations on urethane encouraged extensive studies of its specificity among the carbamates. In general, the more carbamates have been studied the more unique has appeared the effect of urethane against mouse leukemia (Skipper and Bryan, 1949) and against mouse sarcoma 180 (Stock *et al.*, 1953). Against the Walker carcinoma, however, two carbamates other than urethane, the ethyl and the isopropylphenyl carbamates, were active (Haddow and Sexton, 1946). Urethaen has been found to be carcinogenic (Nettleship and Henshaw, 1943; Jaeff, 1947), and this property of urethane appears to be as unique among carbamates as its antileukemia action (Larson, 1947, 1948). Some of the other activities of urethane, however, are shared with other carbamates, e.g., hypnosis (Goodman and Gilman, 1941), mitotic inhibition (Cornman, 1950), and leucopenic action (Skipper *et al.*, 1948).

Bryan *et al.* (1949) have studied urethane with C^{14} labeling in different parts of the molecule in an effort to learn something of the mechanism of its action. Their experiments revealed that 90% of the C of carbonyl group is excreted as CO_2 within 24 hours. It was noted that malignancy bearing animals showed a delayed loss of the labeled carbonyl group. Other experiments have suggested an interference by urethane in the incorporation of formate carbon into nucleic acids (Skipper, 1950).

E. *Other Compounds.* For one reason or another it has not been possible to include all categories of compounds that have been studied. Some, such as the nitrofurans, malononitriles, and stilbenes (Haddow *et al.*, 1948b), have been reviewed by Truhaut (1952), who has included an excellent bibliography on experimental cancer chemotherapy. Other types of compounds may also be found in the Supplement No. 1 of *Cancer Research*, 1953, and the publication of Dyer (1949).

2. Materials of Natural Origin

A. *From Animals.* (a) *Spleen preparations.* Reports on the effectiveness of spleen preparations constantly recur. The studies have had some

basis in reports of the scarcity of primary neoplasia and metastases in the spleen. Reference has been made to Lewisohn's studies (1938). Amersbach *et al.* (1948) have tested intradermal infiltrations of deproteinized extracts from beef, lamb, and human spleens on human malignancies. Thirty out of 46 cases are reported to have shown complete regression. Fardon *et al.* (1948) have observed repression of mitosis in the crypts of Lieberkuhn of mice given injections of beef spleen extract. Diller and Watson (1949) found aqueous extracts of spleen given intraperitoneally or subcutaneously inhibited sarcomas 37 and 180. Nutini *et al.* (1952) reported inhibitory influence of homologous and heterologous splenic extract on the Brown-Pearce carcinoma in rabbits. It would appear, however, that these reports have been insufficient to promote wide use or study of spleen preparations.

(b) *ACS.* A variant in the use of the spleen is to be found in the preparation of ACS, antireticular cytotoxic serum, e.g., antiserum from a rabbit injected with human spleen. For reviews of the extensive background see (Strauss, 1946; Heiman and Meisel, 1949a; Gellhorn and Jones, 1949). Cancer has been included in the many diseases reported handled by this "cure-all" (Bogomolets, 1939, 1943). He has made the usual claims of reduction in pain, improvement in general condition of the patient and prolongation of life. One report of experimental animal studies stimulated by Bogomolets' work states antiserum to rat spleen will inhibit the growth of Walker rat sarcoma 319 in tissue culture (Pomerat, 1945) but there does not appear to have been any anti-rat serum control. Movitz *et al.* (1949) concluded that their experiments with ACS and the Brown-Pearce carcinoma gave results dependent upon dose level that were consistent with the theoretical considerations behind the use of ACS. Larger doses appeared to enhance the malignancy whereas small doses resulted in tumor inhibition. Heiman and Meisel (1949a,b) found that preparations of ACS did not inhibit the growth of spontaneous mammary adenocarcinomas or transplanted sarcoma 180 in mice nor of rat transplantable tumors; however, the incidence of spontaneous mammary adenocarcinoma in R3 female mice was reduced significantly when the ACS was injected during the pre-cancer age (Heiman and Meisel, 1949c).

(c) *Enzymes.* A number of enzymes of animal origin have been studied to combat experimental and human cancer. Several studies on arginase have represented it as active against experimental tumors (Irons, 1946; Neukomm *et al.*, 1949; Vrat, 1951a,b; Wiswell, 1951) and in man (Irons and Boyd, 1952). Confirmatory studies still are required. Crystalline chymotrypsin has been tested without success in experimental animals or in man (West, 1949; Shimkin and Bierman, 1949a). Hyaluronidase in a crude preparation of bull testes has shown no effect on one experimental

tumor; in a mammary adenocarcinoma there were indications of an increased growth rate and possibly more metastases (O'Flynn, 1950). While Greenstein's studies (1947) would suggest that the more subtle enzymes may be most important, it would appear that among the most obvious enzymes for test are deoxyribonuclease and ribonuclease.

(d) *Urine preparations.* Urine has not been neglected in the search for antitumor substances. The observed activities appear not to have been confirmed either because of lack of attempts or lack of results in numerous attempts. Elsasser and Wallace (1939) reported that urine from a patient with embryonic cancer of the testicle caused abortion in the rabbit and, in the rat, necrosis of Walker carcinoma 256. There appears to have been little consideration of the steroids present.

Miller *et al.* (1939) described substances from urine of leukemic patients which could stimulate the myeloid and lymphoid systems of laboratory animals. Later Turner and Miller (1947) considered they could obtain two substances, one, myelokentric acid from patients with chronic myelogenous leukemia which could induce myeloid metaplasia in test animals and the other, lymphokentric acid with corresponding source and properties. While clinical trials have been conducted with myelokentric acid in eight patients with lymphoblastic leukemia, who are said to have improved (Miller *et al.*, 1947), the supplies of material have been inadequate for critical evaluation and no attempts for independent clinical study of these materials have been reported.

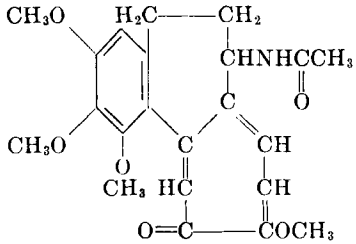
An extract of normal urine designated H11 has been reported to have tumor inhibitory properties (Williams and Walters, 1947). The claims for this material could not be verified in experimental animals (Woodhouse, 1943; Gye *et al.*, 1943) nor in patients (Kidd, 1943). A special committee appointed to review the claims for H11 concluded that its reported activity could not be substantiated (Committee Report, 1948).

(e) *Krebiozen.* The nature of this material is unknown, and its method of preparation from equine blood has not been reported; it merits inclusion as a negative advance because of the failure in its initial evaluation (discussed by Rhoads, 1951) and its lack of activity (Council on Pharmacy and Chemistry, 1951), which coupled with its manner of presentation (Ivy *et al.*, 1951) made it a hindrance to the pursuit of other studies. Unfortunately the nature of the cancer problem is such that until the necessary advances are made there will always be a krebiozen to impede progress.

B. *From Higher Plants.* (a) *Colchicine.* For some time this compound from the autumn crocus has been investigated for antitumor activity because of its effects upon mitosis. New developments on the structure of colchicine (Dewar, 1945) have stimulated additional synthesis on tropolones and other intermediates in the preparation of colchicine; however,

biological data on those compounds have not been published. Andervont (1940) reported observations of tumor hemorrhage from colchicine in confirmation of Boyland and Boyland (1937) and Brues *et al.* (1940). The amount of colchicine required for hemorrhage was found to be close to the lethal dose. Ludford (1945) reviewed studies on colchicine and he (1948) reported that colchicine just below the lethal dose produces hemorrhage which results in tumor regression. He called attention to the similarity of action of colchicine and the bacterial filtrates.

Bass and Feigelson (1948) reported colchicine was more efficient than the nitrogen mustard, HN_2, or urethane in producing acute regression of



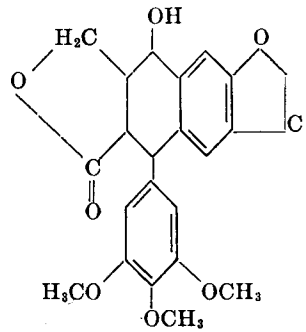
Colchicine

6C3HED lymphosarcomas. In an extension of that study Bass and Probert (1950) showed that this plant material could cause permanent regressions whereas Lits *et al.* (1938) with different conditions had found recurrences of their mouse lymphosarcomas after apparently complete regressions. The results of tumor damage from colchicine already referred to caused Shear *et al.* (1947) to study intensively the diphenylethylamines. This was based upon considerations of the structure of colchicine as it was then understood. Goldberg *et al.* (1950b) have studied colchicine and related compounds for ability to cause gross and histologically evaluated damage to sarcoma 180. These investigators noted that colchicine was the most toxic of the compounds studied and further that toxicity and antineoplastic activity do not change in a parallel manner with structural modifications. Lettré (1950) reported that N-methyl colchicineamide given to patients by iontophoresis was more effective than the parent compound. His studies were based on earlier observations in experimental animals with ascites tumor. A recent report, interesting but presently of obscure significance, is that of Back *et al.* (1951) in which C^{14}-labeled colchicine was administered to normal and tumor-bearing mice. It was then observed that radioactivity was distributed among the normal tissues differing between the two groups of animals only in the lack of radioactivity in the spleens of the tumor-bearing mice.

Although colchicine has given no indication of being a practical thera-

peutic material, it has served to keep investigators aware of the possible value of plants as sources of potentially active materials. Reference has been made to Taylor's studies of extracts of Rain Lily in the embryonated chick egg. Belkin and Fitzgerald (1952, 1953; Belkin *et al.*, 1952; Fitzgerald *et al.*, 1953) have surveyed over 100 plants for ability of their extracts injected subcutaneously to damage mouse sarcoma 37. A large number of these plants in at least one preparation have shown some activity.

(b) *Podophyllin.* Another plant material, podophyllin, from the Mandrake root, was found to possess tumor-damaging properties in a laboratory alert to the potentialities of plant preparations (Hartwell and Shear, 1947). Coincidentally another laboratory accidentally discovered the activity of podophyllin against tumor cells in tissue culture (Ormsbee *et al.*, 1947); however, podophyllin had been scheduled for test because of earlier reports that it caused the production of mitotic figures like those from colchicine (King and Sullivan, 1946). The tissue culture experiments, which initially had employed placental serum contaminated with podophyllin, revealed that crude podophyllin exerts a selective damaging ac-



Podophyllotoxin

tion on mouse tumor cells. Podophyllotoxin did not appear to be as effective as the crude preparation from which it was derived. *In vivo* studies have shown podophyllin causes marked tumor damage accompanied by toxicity to the host (Belkin, 1948).

Shear and his associates investigated podophyllin and found by evaluating fractions for their ability to damage sarcoma 37 that four active compounds were present, α and β peltatins, podophyllotoxin, and quercetin. The last compound was considerably less active than the others (Leiter *et al.*, 1950). Greenspan *et al.* (1950) have reported upon trials of the three most active isolates against lymphomas and other transplantable tumors. Leukemia 1210, lymphosarcomas 1 and 2, mammary adenocarcinoma C3HBA, melanoma S91 in mice, and rat carcinoma 1643 were all damaged extensively by single subcutaneous injections. Complete tumor

regression was not obtained. There appeared to be a large range between the minimum effective and maximum tolerated doses though this varied with the type tumor and host. Clinical trials, not particularly encouraging, have been reported for α peltatin (Greenspan *et al.*, 1952).

C. *From Microorganisms.* The capacity of microorganisms to synthesize many diverse and interesting compounds is attested to by innumerable scientific publications and many products required for daily use. If previously this had not been widely recognized, the practical developments of the various antibiotics must have removed any doubt. The discovery of the remarkable capacities of the antibiotics gave much needed encouragement to attempts already in progress to find substances of microbial origin that would attack cancer cells (see review by Reilly, 1953).

(a) *Bacterial polysaccharides.* Many varieties of organisms or their products have been described as useful in therapy of cancer since the earliest report of Busch (1868). Perhaps the most concentrated studies have been concerned with Coley's toxins, which have been investigated since the latter part of the last century (see reviews by Nauts *et al.*, 1946, 1953). Interest in the action of bacterial preparations on tumors was revived by Gratia and Linz (1931), who observed hemorrhage in the tumor when they injected an *Escherichia coli* filtrate into guinea pigs bearing a transplanted liposarcoma. Confirmation and extensions of their results led Shear and Andervont (1936) to prepare from *E. coli* a polysaccharide capable of producing hemorrhage at very small doses in transplanted mouse sarcomas. Subsequently, *Serratia marcescens* was studied as the source of a hemorrhage-producing polysaccharide. This organism was selected because "it is employed in Coley's toxins; it produces hemorrhage in mouse sarcomas; it is non-pathogenic" (Shear and Turner, 1943). Very small amounts (0.1 μg) of purified polysaccharide obtained in this program are capable of causing hemorrhage in mouse tumors (Shear and Turner, 1943; Hartwell *et al.*, 1943; Kahler *et al.*, 1943). A survey of a large number of bacterial species has indicated that the ability to produce hemorrhage is generally possessed by gram negative bacteria whereas nearly all gram positive ones tested lack that ability (Zahl *et al.*, 1942, 1945; Hutner and Zahl, 1943). Recently a careful study on the isolation of an active material from *E. coli* showed it to be a complex polysaccharide containing a peptide and a phospholipid (Ikawa *et al.*, 1952). The clinical studies of the polysaccharides from *S. marcescens* have continued with occasionally interesting results but the marked toxicity of the preparations has been a deterrent to extensive clinical studies (Brues and Shear, 1944; Reimann and Nishimura, 1949). The extent of the investigations on materials from *E. coli*, *S. marcescens*, and others capable of producing tumor hemorrhage is indi-

cated by a bibliography of at least seventy-five publications from the laboratory of Shear and from others. The unsatisfactory characteristics of the polysaccharide, high toxicity and antigenicity, have stimulated efforts to obtain by various methods modified preparations or fractions with more favorable properties (Creech *et al.*, 1948a,b). Rumors of limited success in this area do not yet appear to have been matched by published data.

(b) *Trypanosoma cruzi studies.* Roskin (1946) has summarized his studies with *Trypanosoma cruzi*, showing that infections with this organism reduced the growth and sometimes caused regressions of Ehrlich carcinoma in mice. Because of the problem of Chagas' disease (of which the mice eventually died), a toxin, KR6, later was prepared by extracting the protozoa. After experiments in mice with Ehrlich carcinoma and with rats bearing the Flexner-Jobling carcinoma, this was used in 60 patients with reported success in some (Klyueva and Roskin, 1946). Hauschka *et al.* (1947) in careful experiments found that infections of *T. cruzi* could retard the growth of certain mouse tumors but that there was extensive parasitization of vital organs with subsequent death of the tumor bearing mice. Cancer cells rarely were parasitized. A toxin prepared from heat killed cells was ineffective. Malisoff (1947) reported confirmation of the Russian findings, but it later appeared his results were to be questioned on the basis of poor tumor material. Hauschka and Goodwin (1948) in extensions of their studies used 5 types of mouse tumors and 8 different strains of *T. cruzi* without obtaining tumor destruction from the K-R factor preparation.

(c) *Other preparations.* Not only has penicillin offered a stimulus to chemotherapy through its benefits against bacteria, but tests of it in crude form against experimental cancer have also provided some provocative results. In tissue culture it was noted that the crude material caused damage to rat or mouse sarcoma cells at concentrations tolerated by normal epithelial cells (Cornman, 1944a,b). Lewis (1944) observed that, while crude material possessed this action, purified penicillin had no effect upon tumor growth in tissue culture. This also was confirmed by Gey *et al.* (1945). *In vitro* experiments provided similar results (Meyer, 1945; Stock *et al.*, 1949b). As usual the *in vitro* results have not been matched *in vivo*. Nevertheless suggestions of an anticancer material in crude penicillin appear in experiments *in vivo* from several laboratories (Beard, 1944; Dobrovolskaia-Zavadskaia, 1946; Stock *et al.*, 1949b). Though the effects have not been strong nor consistent it is difficult to dismiss them completely.

Various microorganisms and nearly all the antibiotics, even noncommercial ones, have been studied without observation of a strong activity

(Reilly, 1953). Of 33 antibiotics tested *in vivo* against sarcoma 180, only 5 have shown a slight retarding effect. Only actidione inhibited the tumor at nontoxic levels (Reilly *et al.*, 1953).

Gliotoxin has been reported active *in vitro* against the Brown-Pearce carcinoma and two mouse tumors (Kidd, 1947) and against mouse sarcoma 180 (Stock *et al.*, 1949b). Crude culture filtrates of *Aspergillus fumigatus* were also found to be active *in vivo* against sarcoma 180. An extension of the studies confirmed the *in vivo* action and revealed the active principle as a toxic protein (Reilly and Stock, 1951): however, its toxicity, the production of focal necrosis of muscle and kidney damage, has thus far discouraged clinical trial. It should be pointed out that the effect against the mouse tumor is not a result of overall toxicity for many hundreds of culture filtrates have been tested at toxic dose levels without any tumor inhibition (Stock and Reilly, unpublished).

It would appear that thus far success of the microbial agents against tumors has been conspicuously lacking. A recent development (Stock *et al.*, unpublished), however, suggests this approach may become a useful one. In an empirical testing of microbial products, a culture filtrate produced by a soil microorganism under laboratory conditions was found to cause a marked inhibition of sarcoma 180 in the mouse without initial or delayed toxicity. Other mouse tumors have been affected but apparently not as greatly as sarcoma 180. Line I leukemia has been considerably inhibited in some mice and a few have been protected indefinitely. The antibiotic preparation has shown a greater toxicity for sarcoma 180 than for normal tissues in tissue culture. If the antibiotic is added to the chorioallantoic membrane of the developing chick embryo, its development becomes abnormal. It exhibits a relatively elongated neck, malformation of the lower beak, and poor bone development. The material has been used without benefit in a few cases of acute leukemia, but there appears to be some benefit in the first group of Hodgkin's disease patients tested with crude and crystalline material. Amounts thus far have been inadequate for trial against other human cancer. While this material is not curative, its marked inhibitory effects on sarcoma 180 and the benefit seen in some cases of Hodgkin's disease are believed more than adequate to lift this approach from the category of pursuit of a will-o-the-wisp.

D. *Viruses.* There has been a reawakening of interest in the growth of viruses in neoplastic tissue (Turner and Mulliken, 1947, 1950; Moore 1949a,b, 1951a,b; Sharpless *et al.*, 1950; Koprowski and Norton, 1950). The early observations, reviewed by Moore (1949a), revealed the survival or actual multiplication of viruses in various tumors, e.g., neurotropic vaccinia in tumors of mice and rats (Levaditi and Nicolau, 1923), vaccinia and Virus III in the Brown-Pearce epithelioma (Rivers and Pearce, 1925)

rabies virus in the Brown-Pearce carcinoma (Schoen, 1937), etc. In most instances there appeared to be no detrimental effect upon the tumors; however, Andrewes (1940) reported premature regressions of the Shope fibroma contaminated with Virus III. Levaditi and Nicolau (1923) indicated difficulty of successful transplants from some tumors infected with vaccinia virus and Levaditi and Haber (1937) found that a mouse adapted strain of avian pest virus inoculated directly into an epithelial tumor, or intraperitoneally, caused massive necrosis of the tumor observed at time of death of mice 3–5 days later.

Such studies have formed the basis for contemporary, practical trials of viruses as experimental cancer chemotherapeutic agents. It is believed that viruses as intracellular agents might be "capable of interference with cell metabolism at fundamental levels perhaps otherwise inaccessible" (Turner and Mulliken, 1947). These investigators found that sarcoma 180 was parasitized and affected in its growth by mouse brain adapted vaccinia virus. A high titer was revealed in the implanted tumor one day after exposure to the virus either *in vitro* or from intratumoral injections. The infected tumors grew more slowly and less successfully. The investigators recognized the objections to these experiments as useful procedures and subsequently extended the studies to include intravenous injections of virus into mice with leukemia or sarcoma 180 (Turner and Mulliken, 1950). The latter appeared uninfluenced in growth though virus localized in the tumor; however, only a low titer of virus developed. There was no effect on two myeloid leukemias and a slight effect on lymphatic leukemia 9417 only when in its subacute form.

In another laboratory the viruses of influenza A, herpes simplex, and Russian Far East encephalitis were tried on transplantable mouse sarcoma 180 (Moore, 1949a,b). Only the latter virus showed a damaging action on the tumor. In this the action was most dramatic. There was early concentration of virus in the tumor, far higher than in brain (Fig. 8), and destruction of the tumor as judged by negative bioassay and histological examination. Unfortunately, this was followed by death of the host from the virus infection for which there is, as yet, no cure. These studies were extended to other viruses and other tumors after further examination of the effects of the Russian virus upon sarcoma 180 (Moore and O'Connor, 1950). Moore (1951b) found that the Russian virus is capable of destroying five other mouse tumors, mammary adenocarcinoma EO771, sarcoma T241, neuroblastoma C1300, fibrosarcoma MCI, and Ridgway osteogenic sarcoma. It was revealed by Kroprowski and Norton (1950) in tests of fifteen neurotropic viruses against six transplantable mouse tumors that it is unwise to generalize on the behavior of viruses in relation to all transplantable tumors. They and other workers have found that viruses differ

in that some will grow in a tumor and destroy it, others will grow readily but have no effect on tumor growth, and still others fail to grow (Moore, 1952). Southam *et al.* (1951) reported that the West Nile and Ilheus encephalitic viruses in mice with AK4, AK4R, or AK9421 leukemia caused a delay in leukemic leukocytosis and infiltration but failed to increase significantly the survival time. Buckley *et al.* (1951a) reported another neurotropic virus, St. Louis encephalitis, has a limited, adverse

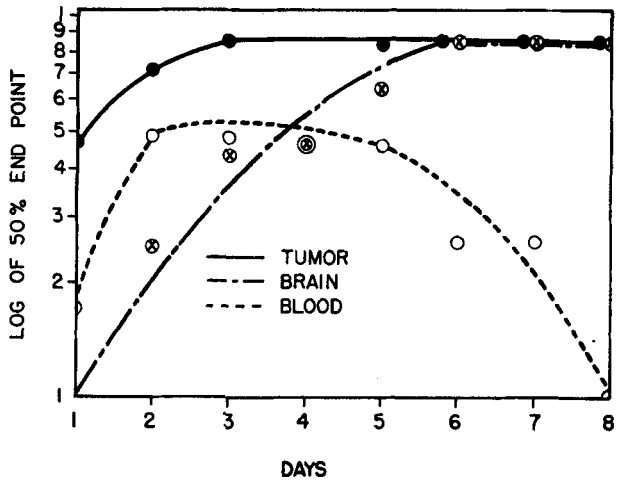


FIG. 8. Relative titers of Russian encephalitis virus in brain, blood, and sarcoma 180 at various intervals after inoculation of the tumor-bearing mouse with virus. (Courtesy of A. E. Moore and *Cancer*.)

effect on the growth and viability of sarcoma 180. The ability of some viruses to destroy certain mouse tumors offers a possible explanation that the loss of tumor strains without detectable bacterial infection may have been due to undetected virus infections. Based on Moore's experiments, Sharpless *et al.* (1950) tested the effects of certain neurotropic viruses on a lymphoid tumor in chickens. In addition to the Russian spring-summer encephalitis virus a number of other viruses and rickettsia were tried. The RPL-12 transplantable lymphoid tumor of chickens could be caused to regress, without apparent damage to the host, by superimposed innoculations of Russian spring-summer, West Nile, Japanese B encephalitis, St. Louis encephalitis, or louping-ill viruses. Ginder and Friedewald (1951) found Semliki Forest virus given to rabbits with induced fibromas caused rapid necrosis and destruction of the tumor.

Although it was recognized that the clinical trial of viruses against cancer would be subjected to special problems in their handling, it was believed that the animal experiments justified an early start on an ex-

tended clinical trial. Southam and Moore (1951) initiated studies with West Nile, Ilheus, and Bunyamwera viruses because they were: (1) oncolytic for experimental tumors, (2) infectious for man, (3) apparently not too dangerous as is the Russian virus, and (4) not likely to have been experienced by many in the general population with resultant development of immunity. The clinical courses of the infections were described. In spite of localization of each of the viruses in tumor tissue of some patients there was no significant effect on the growth of the neoplasms. In a second report Southam and Moore (1952) presented the results of added tests, particularly with Egypt 101 virus. In 4 and possibly 9 of 27 patients in whom infection was established the virus appeared to have a transient inhibitory effect on tumor growth. The authors cite the seemingly almost overwhelming obstacles to practical clinical use of viruses for cancer therapy. First, the patient becomes immune in a short time after receiving the virus. Second, it is necessary to kill all the cancer cells before complete obliteration of the disease can be expected. (Unfortunately this appears to be true in human cancer regardless of the form of therapy.) Third, tumors vary greatly in ability to localize the virus and in their susceptibility to the virus. Fourth, patients differ in their susceptibility to infection and, fifth, although relatively innocuous viruses have been used, there is always the danger of a fatal or disabling reaction in a highly susceptible individual.

Other clinical observations have been reported. Several (Hoster *et al.*, 1949; DePace, 1912) represent chance observations of beneficial effects in cancer that might be attributable to concurrent virus infections. Other investigators stimulated by the animal studies conducted limited trials. Bierman *et al.* (1950) reported a transient hematologic improvement in acute human leukemia following inoculation with feline agranulocytosis virus. Pack (1950) noted unexpected improvements in 2 of 19 melanoma patients following inoculations with rabies vaccine.

It is a question for future decision whether the clinical use of viruses can be made practical. The approach is a fascinating one from a theoretical basis and in the practical results that may be possible. The major difficulties have been mentioned above. The essential requirement is a virus that selectively damages all tumor cells in a host without causing a fatal or disabling infection. Several paths may be taken in the search for the means of successful virus therapy of cancer.

1. A variant of an effective virus, such as the Russian virus, might be found which gives a mild disease in man while retaining ability to damage cancers. The Yellow fever variant found for safe immunization, and experiments which have increased the oncolytic effect of the Russian virus illustrate this possibility (Moore, 1951c). Adaptation of virus in tissue

culture or heterologous growth of human cancer may be employed (Toolan and Moore, 1952).

2. A new suitable virus different from those tested thus far may be found.

3. A viricidal agent effective in controlling a lethal virus infection might be developed which could save a patient after the virus had acted upon the tumor cells. This prospect does not appear particularly bright for it has been considered by many that the virus and cancer problems are so closely alike as to be practically the same; therefore, it probably will be as difficult to find a viricidal agent as a cancer chemotherapeutic agent.

Despite the difficult task in following any of the above experimental objectives to a successful outcome it would appear to be well worth a concentrated effort along each line.

IV. Miscellaneous

1. Special Aids

Brief reference should be made to several compilations of data which have no doubt facilitated and should continue to aid the conduct of chemotherapy studies. The first of these is the collection of chemotherapy data by Dyer (1949) listing all published results meeting certain selected criteria. Supplementing this in 1952 was the publication in preliminary form by *Cancer Research* of negative data from chemotherapy studies. The large amount of available data of this type required issuance of Supplement 1 of *Cancer Research* 1953, which includes negative data on approximately 2000 compounds. The publication of these negative data provide information upon the availability of the compounds, an approximation of their toxicity under definite conditions and the negative results obtained in one or more tumor tests. It is hoped that the information contained in the negative data and the Dyer publications will save much unnecessary duplication of effort. Another publication may assist both the neophyte and the more experienced investigator through perplexing problems of nomenclature. This results from the work of the Committee on Standardized Nomenclature for Inbred Strains of Mice (1952), which has issued a compilation of mouse strains with standardization of nomenclature and a listing of synonyms. One of the most useful of the publications should be that of Dunham and Stewart (1953), who have assembled a list of available transplantable tumors of various species. Included are the characteristics of the tumors most pertinent for those contemplating their experimental use.

2. Resistance in Cancer Cells

One of the most important recent developments has been the demonstration in experimental animals of what in one way had been observed repeatedly in patients and could have been anticipated by analogy from bacteriological data. This was the observation made in mouse leukemia independently by Burchenal *et al.* (1950d) and Law and Boyle (1950) that preparations of variant cells could be obtained which were quite resistant to the chemotherapeutic agents to which preparations of their ancestors were sensitive. In some instances (Law, 1951) the cells not only were resistant to the chemotherapeutic agent but there could be demonstrated a dependence upon the drug. Some will view this development as the realization experimentally of another interesting parallel between cancer cells and bacteria, but to others it will be depressing for the bleak outlook it may give them on the prospects for successful cancer chemotherapy. For the latter there is a ray of hope in the observations of Burchenal *et al.* (1951c) who found that though AK4R leukemia is resistant to the action of five different 4-amino folic acid derivatives it remains sensitive to the crude x-methyl folic acid antagonist. It is also encouraging to note that some leukemic patients who have become resistant to antifolic acid therapy may respond to treatment with 6-mercaptopurine (Burchenal *et al.*, 1953a,b).

3. Combination Therapy

Combinations of two or more materials must be considered as long as no one chemotherapeutic substance is adequate when administered in the best possible way. That the blind use of combinations can lead to difficulties has been emphasized. An editorial (Lancet, 1952) has cited studies on combinations of antibiotics in which it has been shown there may result synergism, no gain, and even antagonism (Jawetz *et al.*, 1951a,b; Speck *et al.*, 1951; Jawetz and Gunnison, 1952).

Combination therapy has not been employed extensively in cancer chemotherapy though the need for it is obvious. Possibly this situation arises for the reasons that relatively few chemotherapeutic agents are available, that they have been developed comparatively recently, and that they are effective only at toxic or near toxic levels. Also the permutations in combination therapy are so numerous as to make one pause before embarking upon a program of that nature. Skipper (1949) in trials of the nitrogen mustard HN_2 and urethane was able to demonstrate a small gain in effect against mouse leukemia. Subsequently Skipper *et al.* (1951a) tested combinations of other known antileukemic agents to learn whether

any of them in combination produced additive effects. Most of the studies were devoted to attempts to improve the effectiveness of aminopterin. Only the combination of urethane with nitrogen mustard exhibited evidence of synergistic activity. Emerson *et al.* (1950) have reported superior results when riboflavin deficiency was coupled with the administration of steroids which alone are capable of only partial control of the mouse lymphosarcoma used in the study. Methyl testosterone or cortisone given to the pyridoxine-deficient rat increases the inhibition of a rat lymphosarcoma produced by either steroid alone (Ballantyne and McHenry, 1949). Shapiro and Gellhorn (1951) have employed combinations of 8-azaguanine with either deoxypyridoxine, folic acid, 7-methyl folic acid, or vitamin B_{12} against mouse mammary adenocarcinoma 755. The combinations provided greater inhibition of the tumors than any of the drugs used alone. Extensions of these studies (Fugmann and Shapiro, 1952; Shapiro and Fugmann, 1952; Shapiro *et al.*, 1952; Shapiro, 1952) included studies of 8-azaguanine with flavotin, a riboflavin antagonist, with 6-formyl pteridine, and with sex hormones. In the last studies the inhibitory effect of diethylstilbestrol on 755 mammary adenocarcinoma in C57BL mice was considerably greater when 8-azaguanine was used with it. 8-Azaguanine and other agents, including α peltatin, aminopterin, TEM, HN_2 were studied in combination against the transplantable acute lymphoid leukemia, L1210 by Goldin *et al.* (1952). Evidence of additive effects were observed with the combinations; the best combinations varied depending upon the method of evaluation whether increased survival time, inhibition of local tumor growth, or response of peripheral blood. Law (1952) also has reported combinations of antileukemic agents against acute lymphoid leukemia in mice. Amethopterin and 8-azaguanine together were reported to show a striking potentiation.

Combinations of antitumor agents of limited usefulness merit trial; however, it does not seem desirable to limit the combinations to those compounds which have shown antitumor activity. In following an approach with a firm theoretical basis, that Potter (1951) has termed sequential blocking, it may be necessary to test tumor inactive compounds in combinations with those that are inhibitory. The end result of sequential blocking of enzymes would be similar to that from a series of inefficient organic syntheses. Any organic chemist readily appreciates the small overall yield that would result if the product at any step in a series of syntheses is no more than 50% of theory. This device might well prove to be the key to a more effective chemotherapy in any area. Thus attempts should be made to apply sequential blocking by use of a number of substances capable of interfering with several enzymes at various steps in a

metabolic pathway or in another approach by interference with enzymes in multiple alternate pathways. A logical start would be an attempt to interfere with nucleoprotein metabolism.

The combination of certain drugs with x-rays and even surgery appears warranted. Mitchell (1948) based upon tissue culture observations of mitotic inhibition of chick fibroblasts tried combination of parenterally administered synkayvite (tetrasodium 2-methyl-1,4-naphthohydroquinonediphosphate) with x-ray therapy. He reported increased survival time in those patients so treated compared with those given only x-rays. Gellhorn and Gagliano (1950) were inclined to question these results in view of the absence of any indication of tumor inhibitory or morphological damaging action of synkayvite upon five transplantable tumors in experimental animals.

Palliation of liver metastases has been obtained by the use of nitrogen mustard (given intravenously with three limbs protected by tourniquets) followed by doses of x-rays ranging from 2000 to 3750 r given at 1000 kv to the whole liver in 8 days. Good symptomatic improvement was obtained in approximately three-fourths of the patients; liver function studies performed before or during and at intervals after treatment likewise showed improvement or no impairment. However, the limited number of patients does not permit an evaluation of the effectiveness of the treatment relative to x-ray alone (Phillips *et al.*, in press; Hamilton, to be published).

4. Other Improvements in Use of Agents

A. *Intra-arterial Administration.* Another attempt to improve the benefit from agents of limited usefulness has been the development of techniques for intra-arterial administration of drugs, particularly nitrogen mustard HN_2. Introduction of the mustard into the arterial supply of tumors of head and neck and pelvis has resulted in temporary regressions and symptomatic improvement. Larger doses of HN_2 have been tolerated. Several deaths with symptoms suggesting electrolyte disturbance raised the question of factors other than hematologic as limitations of localized HN_2 therapy (Klopp *et al.*, 1950; Bateman *et al.*, 1951). Additional studies on this subject include Bierman *et al.* (1951a,b), Cromer *et al.* (1952), Sullivan *et al.* (1953a,b), Bateman *et al.* (1953), and Barberio *et al.* (1953).

B. *Alteration of Agents.* As more antitumor substances, varied in chemical type and possible mode of action, become available, the possibility of alternation among the several chemotherapeutic substances will increase so that a patient developing resistance to one form of chemotherapy may be shifted to another drug with some expectation of renewed

benefit, somewhat as has been occasionally possible in shifts from radiation to chemotherapy. Studies with corticotropin, cortisone, and some folic acid antagonists in leukemia illustrate this. In those experiments it was considered that 50% of the patients resistant to one form of therapy responded to the alternate form of chemotherapy (Kingsley-Pillers *et al.*, 1952). 6-Mercaptopurine may prove useful as an alternate to the folic acid analogs (Burchenal *et al.*, 1953b).

5. Radioactive Isotopes

The use of radioactive isotopes in general belongs in a discussion of radiation rather than chemotherapy. Much of the use of isotopes in cancer represents a convenient, nonspecific application of radiation, for example against ascites tumor cells (Goldie *et al.*, 1951; Williams and Williams, 1952; Lewin *et al.*, 1952) and mouse leukemia (Hahn *et al.*, 1951). However, in a few instances the nature of the use of radioactive isotopes brings them within the scope of experimental chemotherapy, for example when the localization of the radioactivity within the animal depends upon some chemical characteristics and possibly certain biological specificities. Perhaps the first and best example of this is the use of radioactive iodine for cancer of the thyroid (Keston *et al.*, 1942; Rawson *et al.*, 1949). Another approach, with possibilities of specific localization of radioactivity by immunological reaction, is to be found in the preparation of specific tissue antibodies with subsequent addition of radioactive iodine by chemical reaction with the antibodies (Pressman, 1949). Such preparations will be of little more than theoretical interest unless they can be freed of antibodies to nonspecific tissue antigens. The localization of injected radioactive dyes in tumors appears to be nonspecific and dependent upon conditions in the blood supply for any temporal apparent specificity (Shapiro and Landing, 1948); however, Sloviter (1949) has reported localization of radioactive (I^{131} labeled) Nile Blue 2B in mouse tumors. The localization in the kidney, liver, and spleen was of the same order of magnitude as that in the tumor. Prolongation in survival of the mice was significantly greater when the radioactive dye was used. Stevens *et al.* (1952) has suggested the possibility of precipitating substances in tumor tissue based upon differences in solubility of the substances at the different pH's of blood and the interior portion of the tumor. Use of this approach might be another method of localizing radioactivity but possibly would fail through limitation in the amount of precipitated radioactivity in the outer portion of the tumor which would allow too many tumor cells to escape treatment.

The greatest use of radioactive isotopes in experimental cancer chemotherapy would appear to be through the same role it is playing in so

much of modern biochemistry. This is the use of tracers in metabolism studies to provide a better understanding of the mode of action of compounds now known to have antitumor activity and to provide a better basis for the rational selection of compounds for future study. Skipper and associates have used a number of labeled compounds to study the mode of action of chemotherapeutic agents. Reference has already been made to the studies with labeled urethane (also Skipper *et al.*, 1951b). Using the technique of measuring the incorporation of C^{14} of formate into nucleic acid purines in mice it was found that aminopterin and amethopterin inhibit that incorporation (Skipper *et al.*, 1950c) as do 2,6-diaminopurine, 8-azaguanine, cortisone, potassium arsenite, urethane, and nitrogen mustard (Skipper *et al.*, 1951c). C^{14} was found in nucleic acid fractions of mammary adenocarcinoma EO771 and normal tissues of mice given 8-azaguanine labeled in the 2 position (Mitchell *et al.*, 1950). There appeared to be no preferential accumulation or fixation in the tumors (Bennett *et al.*, 1950). Carló and Mandel (1953) found that the administration of 8-azaguanine made no decisive difference in incorporation of guanine-$4C^{14}$ or 4-amino-5-imidazole carboxamide into tumor or liver.

6. General Considerations

From what has gone before it is obvious that useful but not curative agents against cancer have been found. The steroids, the carbamates, folic acid analogs, the nitrogen mustards, and related compounds are the most effective. While it can be anticipated that additional, active antifolic acids and nitrogen mustard-like compounds can be found, it is difficult to escape the impression that studies in all four categories of compounds have reached the point of diminishing returns as far as providing new compounds with superior degrees of effectiveness. Therefore, new types of agents and better ways of utilizing those at hand must be sought. In this search consideration must be given to the limitations inherent with any substance that acts merely upon rapidly proliferating tissue by virtue of its chemical reactivity or because it may interfere in the metabolism of normal tissue as well as neoplastic cells. Philips (1950) has reviewed the evidence indicating that the effects of the mustards are readily apparent on susceptible rapidly proliferating tissue whether normal or abnormal. Skipper *et al.* (unpublished) in studies on the incorporation of C^{14} formate in various normal tissues and a number of tumors in mice have found that certain normal tissues, for example jejunum, are as active metabolically as the tumors. Thus any simple interference in a metabolic process common to tumors and normal tissue would be limited as a therapeutic procedure by the damage caused to the most sensitive normal tissues. Success in the antimetabolite approach

may come by a chance discovery or when qualitative or sufficiently large quantitative differences are discovered through fundamental studies.

Differences in the metabolism of normal and tumor cells may provide a basis for eventual success with viruses against tumors. Some of the present observations on tumor destruction by viruses are the result of the marked support for virus multiplication in the rapidly growing tumor tissue and some is due to the selective infectivity of the viruses. Increase in that selectivity appears an essential factor in future success of this approach.

The question of the influence in overall toxicity in the effects of chemotherapeutic agents has been raised by Lees and Lees (1950) and has been a matter of concern by various investigators who have used tumor bearing animals in chemotherapy experiments (Haddow *et al.*, 1937; Goldin *et al.*, 1949b; Stock, 1950; Walpole, 1951; Bennette, 1952). While effective compounds have been toxic, most of them in decreased, well-tolerated doses have shown a decreased but definite antitumor activity; furthermore, probably all the investigators referred to above and many others have seen numerous compounds that have exhibited no apparent antitumor activity even at highly toxic doses. The effect of compounds upon food intake has been a problem which has required attention. In careful studies Tannenbaum (1947) has determined the effect of reduced caloric intake on the reduced incidence and growth of tumors. Others have investigated the influence of other dietary restrictions (King *et al.*, 1947).

The discovery by mouse and rat tests of substances useful in human cancer should provide an answer to the frequently raised question of whether results can be transferred from mouse to man. Actually, within limits, it may be more difficult to transfer results from one mouse tumor to another than from experimental animals to man. This has been emphasized by studies in a spectrum of experimental tumors which illustrate the desirability of testing as broadly as possible compounds of theoretical interest and also of testing against all forms of human malignancy any material found active in well-tolerated doses against any animal tumor.

In the next period of time equal to the 6 to 10 years chiefly covered by this chapter substances can be expected that will overshadow those reported here. With some luck and considerable effort these expectations may be realized in one or more of the following ways:

1. Continued empirical screening of natural materials and testing with an increased rationale in the selection of compounds based upon the accumulating data from empirical studies. Important in considerations of materials for study undoubtedly will be those capable of interfering with nucleic acid metabolism. Several simple materials in this category

have shown activity. The more complex nucleosides and nucleotides merit study.

2. Development of additional antimetabolites.

3. Production of more and better antitumor antibiotics.

4. Better use of presently available agents, and those to come, by combinations with radiation or by combinations of agents, particularly in combinations with a rationale.

5. Study of agents against human cancer growing in a heterologous host.

6. Use of viruses or other infectious agents as therapeutic procedures.

V. Summary

In this chapter an attempt has been made to consider the compounds, other materials, and test methods for their detection, which have occupied the attention of investigators in experimental cancer chemotherapy mainly during the period since 1945. Briefly outlined were typical techniques in tissue culture, with embryonated eggs, of mouse leukemia, and with animals bearing various solid tumors. Some of these procedures, particularly those employing animals with malignancies, are capable of selecting materials of potential clinical usefulness.

Although no human cancer cures are available, a few steroids, some folic acid analogs, the nitrogen mustards, and related compounds are definitely of use in certain forms of human cancer. Among the other compounds, we have considered some which lack sufficient activity at tolerated doses, some which have not been adequately examined, and some which hold promise for continued interest and potential usefulness. Though the advances covered in this chapter have been considerable, their limited extent is recognized. There appears to be no strong basis at present for prediction of the discovery of a cancer cure within the next few years; however, one can with confidence forecast that the next few years will bring advances in experimental cancer chemotherapy which will reveal those discussed in this chapter to be the few feeble steps which they are.

REFERENCES

Adair, F. E., and Bagg, H. J. 1931. *Ann. Surg.* **93,** 190–199.

Adair, F. E., Mellors, R. C., Farrow, J. H., Woodard, H. Q., Escher, G. C., and Urban, J. A. 1949. *J. Am. Med. Assoc.* **140,** 1193–1200.

Alexander, P. 1952. *Nature* **169,** 226–227.

Alexander, P., and Fox, M. 1952. *Nature* **169,** 572–574.

Alexander, P., Fox, M., Stacey, K. A., and Smith, L. F. 1952. *Biochem. J.* **52,** 177–184.

Ambrus, C. M., Ambrus, J. L., Harrisson, J. W. E., and Cravetz, H. 1951. *Brit. J. Cancer* **5,** 311–316.

Amersbach, J. C., Walter, E. M., and Sperti, G. S. 1948. *Arch. Dermatol. and Syphilol.* **58,** 111–117.
Andervont, H. B. 1940. *J. Natl. Cancer Inst.* **1,** 361–366.
Andrewes, C. H. 1940. *J. Pathol. Bacteriol.* **50,** 227–234.
Aposhian, H. V., and Lambooy, J. P. 1951. *Proc. Soc. Exptl. Biol. Med.* **78,** 197–199.
Armistead, G. C., Jr., Burchenal, J. H., Karnofsky, D. A., and Southam, C. M. 1949. *Cancer* **2,** 1087–1088.
Back, A., Walaszek, A., and Uyeki, E. 1951. *Proc. Soc. Exptl. Biol. Med.* **77,** 667–669.
Ballantyne, R. M., and McHenry, E. W. 1949. *Cancer Research* **9,** 689–691.
Barberio, R., Berry, N., Bateman, J., Cromer, J. K., and Klopp, C. T. 1953. *Cancer* **6,** 283–287.
Barker, G. M., Dhar, M. M., and Parsons, L. D. 1949. *Brit. J. Cancer* **3,** 427–429.
Barker, G. M., Dhar, M. M., and Parsons, L. D. 1951. *Brit. J. Cancer* **5,** 124–129.
Bass, A. D., and Freeman, M. L. H. 1946. *J. Natl. Cancer Inst.* **7,** 171–176.
Bass, A. D., and Feigelson, M. 1948. *Cancer Research* **8,** 503–509.
Bass, A. D., and Probert, C. 1950. *Cancer Research* **10,** 420–422.
Bateman, J. C., Klopp, C. T., and Cromer, J. K. 1951. *Blood* **6,** 26–38.
Bateman, J. C., Cornman, I., Grice, P. M., Kennelly, J. M., Jr., and Klopp, C. T. 1953. *Cancer* **6,** 275–279.
Bauer, K. H. 1949. *Klin. Wochschr.* **27,** 159–163.
Bayrd, E. D., Stickney, J. M., Hall, B. E., and Watkins, C. H. 1952. *Cancer* **5,** 336–343.
Beard, H. H. 1944. *Exptl. Med. Surg.* **2,** 286–289.
Belkin, M. 1948. *J. Pharmacol. Exptl. Therap.* **93,** 18–25.
Belkin, M., and Fitzgerald, D. B. 1952. *J. Natl. Cancer Inst.* **13,** 139–156.
Belkin, M., Fitzgerald, D. B., and Felix, M. D. 1952. *J. Natl. Cancer Inst.* **13,** 741–744.
Belkin, M., and Fitzgerald, D. B. 1953. *J. Natl. Cancer Inst.* **13,** 889–893.
Bennett, L. L., Jr., Skipper, H. E., Mitchell, J. H., Jr., and Sugiura, K. 1950. *Cancer Research* **10,** 644–646.
Bennette, J. G. 1952. *Brit. J. Cancer* **6,** 377–388.
Berman, L., Axelrod, A. R., Vonder Heide, E. C., and Sharp, E. A. 1949. *Am. J. Clin. Pathol.* **19,** 127–133.
Bierman, H. R., McD. Hammon, W., Eddie, B. U., Meyer, K. F., and Shimkin, M. B. 1950. *Cancer Research* **10,** 203–204.
Bierman, H. R., Byron, R. L., Jr., Kelly, K. H., and Cordes, F. 1951a. *Cancer Research* **11,** 236.
Bierman, H. R., Kelly, K. H., Byron, R. L., Jr., Dod, K. S., and Shimkin, M. B. 1951b. *J. Natl. Cancer Inst.* **11,** 891–897.
Biesele, J. J., Philips, F. S., Thiersch, J. B., Burchenal, J. H., Buckley, S. M., and Stock, C. C. 1950. *Nature* **166,** 1112–1113.
Biesele, J. J., Berger, R. E., Wilson, A. Y., Hitchings, G. H., and Elion, G. B. 1951. *Cancer* **4,** 186–197.
Biesele, J. J. 1952. *Cancer* **5,** 787–791.
Biesele, J. J., Berger, R. E., and Clarke, M. 1952a. *Cancer Research* **12,** 399–406.
Biesele, J. J., Berger, R. E., and Clarke, M. 1952b. *Cancer Research* **12,** 465–470.
Biesele, J. J., Berger, R. E., Clarke, M., and Weiss, L. 1952c. *Exptl. Cellular Research* **Suppl. 2,** 279–303. The Chemistry and Physiology of the Nucleus, Academic Press.
Biesele, J. J., *et al.* Unpublished data.
Bischoff, F., Ingraham, L. P., and Rupp, J. J. 1943. *Arch. Pathol.* **35,** 713–716.
Bogomolets, A. A. 1939. *3rd Intern. Congr. Cancer* 132–133.

Bogomolets, A. A. 1943. *Am. Rev. Soviet Med.* **1**, 101–112.
Boyland, E., and Boyland, M. E. 1937. *Biochem. J.* **31**, 454–460.
Boyland, E. 1948. *Biochem. Soc. Symposia (Cambridge, Engl.)* **2**, 61–73.
Boyland, E., Clegg, J. W., Koller, P. C., Rhoden, E., and Warwick, O. H. 1948. *Brit. J. Cancer* **2**, 17–29.
Boyland, E., and Horning, E. S. 1949. *Brit. J. Cancer* **3**, 118–123.
Boyland, E. 1952. *Endeavour* **11**, 87–91.
Browning, H. C. 1949. *Cancer* **2**, 646–672.
Brues, A. M., Marble, B. B., and Jackson, E. B. 1940. *Am. J. Cancer* **38**, 159–168.
Brues, A. M., and Shear, M. J. 1944. *J. Natl. Cancer Inst.* **5**, 195–208.
Bryan, C. E., Skipper, H. E., and White, L., Jr. 1949. *J. Biol. Chem.* **177**, 941–950.
Brzustowicz, R. J., Svien, H. J., Bennett, W. A., and Higgins, G. M. 1951. *Proc. Staff Meetings Mayo Clinic* **26**, 121–128.
Buckley, S. M., Stock, C. C., Crossley, M. L., and Rhoads, C. P. 1950. *Cancer Research* **10**, 207–208.
Buckley, S. M., Buckley, J. J., and Snipes, A. E. 1951a. *Cancer* **4**, 367–374.
Buckley, S. M., Stock, C. C., Parker, R. P., Crossley, M. L., Kuh, E., and Seeger, D. R. 1951b. *Proc. Soc. Exptl. Biol. Med.* **78**, 299–305.
Buckley, S. M., Stock, C. C., Crossley, M. L., and Rhoads, C. P. 1952. *Cancer* **5**, 144–152.
Burchenal, J. H., Lester, R. A., Riley, J. B., and Rhoads, C. P. 1948a. *Cancer* **1**, 399–412.
Burchenal, J. H. 1948b. *Radiology* **50**, 494–499.
Burchenal, J. H., Myers, W. P. L., Craver, L. F., and Karnofsky, D. A. 1949a. *Cancer* **2**, 1–17.
Burchenal, J. H., Burchenal, J. R., Kushida, M. N., Johnston, S. F., and Williams, B. S. 1949b. *Cancer* **2**, 113–118.
Burchenal, J. H., Bendich, A., Brown, G. B., Elion, G. B., Hitchings, G. H., Rhoads, C. P., and Stock, C. C. 1949c. *Cancer* **2**, 119–120.
Burchenal, J. H., Johnston, S. F., Burchenal, J. R., Kushida, M. N., Robinson, E., and Stock, C. C. 1949d. *Proc. Soc. Exptl. Biol. Med.* **71**, 381–387.
Burchenal, J. H., Babcock, G. M., Broquist, H. P., and Jukes, T. H. 1950a. *Proc. Soc. Exptl. Biol. Med.* **74**, 735–737.
Burchenal, J. H., Crossley, M. L., Stock, C. C., and Rhoads, C. P. 1950b. *Arch. Biochem.* **26**, 321–323.
Burchenal, J. H., Stock, C. C., and Rhoads, C. P. 1950c. *Cancer Research* **10**, 209.
Burchenal, J. H., Robinson, E., Johnston, S. F., and Kushida, M. N. 1950d. *Science* **111**, 116–117.
Burchenal, J. H., Cremer, M. A., Williams, B. S., and Armstrong, R. A. 1951a. *Cancer Research* **11**, 700–705.
Burchenal, J. H., Karnofsky, D. A., Kingsley-Pillers, E. M., Southam, C. M., Myers, W. P. L., Escher, G. C., Craver, L. F., Dargeon, H. W., and Rhoads, C. P. 1951b. *Cancer* **4**, 549–569.
Burchenal, J. H., Babcock, G. M., Armstrong, R. A., and Robinson, E. 1951c. *Acta Unio Intern. contra Cancrum* **7**, 436–442.
Burchenal, J. H., Johnston, S. F., Parker, R. P., Crossley, M. L., Kuh, E., and Seeger, D. R. 1952a. *Cancer Research* **12**, 251–252.
Burchenal, J. H., Goetchius, S. K., Stock, C. C., and Hitchings, G. H. 1952b. *Cancer Research* **12**, 251.
Burchenal, J. H., Karnofsky, D. A., Murphy, L., Ellison, R. L., and Rhoads, C. P. 1953a. *Proc. Am. Assoc. Cancer Research* **1**, 7–8.

Burchenal, J. H., Murphy, M. L., Ellison, R. R., Sykes, M. P., Tan, T. C., Leone, L. A., Karnofsky, D. A., Craver, L. F., Dargeon, H. W., and Rhoads, C. P. 1953b. *Blood* **8**, 965–999.

Busch. 1868. *Berlin. klin. Wochschr.* **5**, 137–138.

Butler, J. A. V., Gilbert, L. A., and Smith, K. A. 1950. *Nature* **165**, 714–716.

Butler, J. A. V., and Smith, K. A. 1950. *J. Chem. Soc.* 3411–3418.

Campbell, J. G. 1949. *Brit. J. Cancer* **3**, 72–88.

Carló, P. E., and Mandel, H. G. 1953. *J. Biol. Chem.* **201**, 343–347.

Carter, T. C., Dunn, L. C., Falconer, D. S., Grüneberg, H., Heston, W. E., and Snell, G. D. 1952. *Cancer Research* **12**, 602–613.

Cartwright, G. E., Palmer, J. G., Hitchings, G. H., Elion, G. B., Gunn, F. D., and Wintrobe, M. M. 1950. *J. Lab. Clin. Med.* **35**, 518–527.

Chute, R. N., Sommers, S. C., and Warren, S. 1952. *Cancer Research* **12**, 912–914.

Clarke, D. A., Buckley, S. M., Sternberg, S. S., Stock, C. C., Rhoads, C. P., and Hitchings, G. H. 1952. *Cancer Research* **12**, 255.

Clarke, D. A., Philips, F. S., Sternberg, S. S., Stock, C. C., and Elion, G. B. 1953a. *Proc. Am. Assoc. Cancer Research* **1**, 9–10.

Clarke, D. A., Philips, F. S., Sternberg, S. S., Stock, C. C., Elion, G. B., and Hitchings, G. H. 1953b. *Cancer Research* **13**, 593–604.

Clarke, D. A., Philips, F. S., Sternberg, S. S., Barclay, R. K., Stock, C. C. 1953c. *Proc. Soc. Exptl. Biol. Med.* **84**, 203–207.

Cohen, A. L., Borsook, H., and Dubnoff, J. W. 1947. *Proc. Soc. Exptl. Biol. Med.* **66**, 440–444.

Colsky, J., Greenspan, E. M., and Schoenbach, E. B. 1952. *Cancer* **5**, 1221–1224.

Committee on Standardized Nomenclature for Inbred Strains of Mice. 1952. *Cancer Research* **12**, 602–613.

Committee Report. 1948. *Brit. Med. J.* **2**, 701–708.

Conway, B. E., Gilbert, L., and Butler, J. A. V. 1950. *J. Chem. Soc.* 3421–3425.

Cornman, I. 1944a. *Science* **99**, 247.

Cornman, I. 1944b. *J. Gen. Physiol.* **28**, 113–118.

Cornman, I. 1950. *J. Natl. Cancer Inst.* **10**, 1123–1128.

Council on Pharmacy and Chemistry. 1951. *J. Am. Med. Assoc.* **147**, 864–873.

Creech, H. J., Hamilton, M. A., and Diller, I. C. 1948a. *Cancer Research* **8**, 318–329.

Creech, H. J., Hamilton, M. A., Nishimura, E. T., and Hankwitz, R. F., Jr. 1948b. *Cancer Research* **8**, 330–336.

Cromer, J. K., Bateman, J. C., Berry, G. N., Kennelly, J. M., Klopp, C. T., and Platt, L. I. 1952. *Am. J. Obstet. Gynecol.* **63**, 538–548.

Crossley, M. L., Allison, J. B., Wainio, W. W., Muenzen, J. B., Parker, R. P., Kuh, E., and Seeger, D. R. 1951a. *Abstr. 12th Intern. Congr. Pure Appl. Chem.* (*New York*) 331–332.

Crossley, M. L., Allison, J. B., Wainio, W. W., and Muenzen, J. R. 1951b. *J. Natl. Cancer Inst.* **12**, 305–321.

Crossley, M. L., Allison, J. B., Parker, R. P., Kuh, E., and Seeger, D. R. 1952a. *Cancer Research* **12**, 256.

Crossley, M. L., Allison, J. B., Parker, R. P., Kuh, E., and Seeger, D. R. 1952b. Abstr. Meet. Am. Chem. Soc., Sept. 19, Atlantic City.

Dameshek, W. 1949. *Blood* **4**, 168–171.

Dameshek, W., Freedman, M. H., and Steinberg, L. 1950. *Blood* **5**, 898–915.

DePace, N. G. 1912. *La Ginecologia* **9**, 82.

DeRenzo, E. C., and Cerecedo, L. R. 1950. *Proc. Soc. Exptl. Biol. Med.* **73**, 356–359.

Dewar, M. J. S. 1945. *Nature* **155**, 141–142.

Diller, I. C., Beck, L. V., and Blauch, B. 1948. *Cancer Research* **8,** 581–585.
Diller, I. C., and Watson, G. F. 1949. *Growth* **13,** 27–44.
Dobrovolskaia-Zavadskaia, N., and Zephiroff, P. 1939. *Compt. rend. soc. biol.* **132,** 352–355.
Dobrovolskaia-Zavadskaia, N. 1946. *Bull. assoc. franç. étude cancer* **33,** 192–222.
Downing, V., Farber, S., and Abu-Hydar, M. 1953. *Proc. Am. Assoc. Cancer Research* **1,** 13.
Dunham, L. J., and Stewart, H. L. 1953. *J. Natl. Cancer Inst.* **13,** 1299–1377.
Dustin, P. 1947. *Nature* **159,** 794–797.
Dustin, P. 1950. *Compt. rend. soc. biol.* **144,** 1297–1299.
Dyer, H. M., and Kelly, M. G. 1946. *J. Natl. Cancer Inst.* **7,** 177–182.
Dyer, H. M. 1949. An Index of Tumor Chemotherapy, 1st ed. U.S. Govt. Printing Office, Washington, D.C.
Elion, G. B., Hitchings, G. H., Stock, C. C., Sugiura, K., and Buckley, S. M. 1952. *Cancer Research* **12,** 259.
Elsasser, T. H., and Wallace, G. B. 1939. *Science* **89,** 250–251.
Emerson, G. A., Wurtz, E., and Zanetti, M. E. 1950. *Federation Proc.* **9,** 357–358.
Emerson, G. A. 1951. *Trans. N.Y. Acad. Sci.* **13,** II, 92–94.
Engstrom, R. M., Kirschbaum, A., and Mixer, H. W. 1947. *Science* **105,** 255–256.
Everett, J. L., and Kon, G. A. R. 1950. *J. Chem. Soc.* 3131–3155.
Falco, E. A., Goodwin, L. G., Hitchings, G. H., Rollo, I. M., and Russell, P. B. 1951. *Brit. J. Pharmacol.* **6,** 185–200.
Farber, S., Cutler, E. C., Hawkins, J. W., Harrison, J. H., Pierce, E. C., and Lenz, G. G. 1947. *Science* **106,** 619–621.
Farber, S., Diamond, L. K., Mercer, R. D., Sylvester, R. F., and Wolff, J. A. 1948. *New Engl. J. Med.* **238,** 787–793.
Farber, S., Diamond, I., Foley, G., and Modest, E. J. 1952. *Am. J. Pathol.* **28,** 559.
Farber, S., Appleton, R., Downing, V., Heald, F., King, J., and Toch, R. 1953a. *Cancer* **6,** 135–141.
Farber, S., Foley, G., Downing, V., Appleton, R., and King, J. 1953b. *Proc. Am. Assoc. Cancer Research* **1,** 15–16.
Fardon, J. C., Prince, J. E., and Berning, N. 1948. *Cancer Research* **8,** 531–533.
Ferguson, F. C., Jr., Thiersch, J. B., and Philips, F. S. 1950. *J. Pharmacol. Exptl. Therap.* **98,** 293–299.
Finkelstein, M., and Thomas, P. A. 1951. *Cancer Research* **11,** 801–806.
Finkelstein, M., Winters, W. D., Thomas, P. A., Davison, C., and Smith, P. K. 1951. *Cancer Research* **11,** 807–810.
Fitzgerald, D. B., Belkin, M., Felix, M. D., and Carroll, M. K. 1953. *J. Natl. Cancer Inst.* **13,** 895–903.
Flory, C. M., Furth, J., Saxton, J. A., Jr., and Reiner, L. 1943. *Cancer Research* **3,** 729–743.
Foley, E. J., and Silverstein, R. 1951. *Proc. Soc. Exptl. Biol. Med.* **77,** 713–715.
Foley, E. J. 1952. *Proc. Soc. Exptl. Biol. Med.* **80,** 669–671.
Freedlander, B. L., and Furst, A. 1952. *Proc. Soc. Exptl. Biol. Med.* **81,** 638–640.
Friedenwald, J. S., Buschke, W., and Moses, S. G. 1948. *Bull. Johns Hopkins Hosp.* **82,** 312–325.
Friedman, O. M., and Rutenburg, A. M. 1950. *Proc. Soc. Exptl. Biol. Med.* **74,** 764–766.
Friend, C. 1951. *Proc. Soc. Exptl. Biol. Med.* **78,** 150–153.
Fugmann, R. A., and Shapiro, D. M. 1952. *Cancer Research* **12,** 263.
Galton, D. A. G., and Timmis, G. M. 1952. *29th Annual Rept. Brit. Empire Cancer Campaign,* 27.

Galton, D. A. G. 1953. *Lancet* **264,** I, 208–213.
Gardner, W. U. 1947. *Recent Progr. Hormone Research* **1,** 217–259.
Gardner, W. U. 1953. *Advances in Cancer Research* **1,** 173.
Gellhorn, A., and Jones, L. O. 1949. *Am. J. Med.* **6,** 188–231.
Gellhorn, A., Engelman, M., Shapiro, D. M., Graff, S., and Gillespie, H. 1950. *Cancre Research* **10,** 170–177.
Gellhorn, A., and Gagliano, T. 1950. *Brit. J. Cancer* **4,** 103–107.
Gellhorn, A. 1953. *Cancer Research* **13,** 205–215.
Gey, G. O., Gey, M. K., Invi, F., and Vedder, H. 1945. *Bull. Johns Hopkins Hosp.* **77,** 116–131.
Gilman, A., and Philips, F. S. 1946. *Science* **103,** 409–415.
Ginder, D. R., and Friedewald, W. F. 1951. *Proc. Soc. Exptl. Biol. Med.* **77,** 272–276.
Goldacre, R. J., Loveless, A., and Ross, W. C. J. 1949. *Nature* **163,** 667–669.
Goldberg, L., Klein, E., and Klein, G. 1950a. *Exptl. Cell Research* **1,** 543–570.
Goldberg, B., Ortega, L. G., Goldin, A., Ullyot, G. E., and Schoenbach, E. B. 1950b. *Cancer* **3,** 124–129.
Goldberg, B., and Schoenbach, E. B. 1951. *Cancer* **4,** 1125–1139.
Goldie, H., Watkins, F. B., Powell, C., and Hahn, P. F. 1951. *Proc. Soc. Exptl. Biol. Med.* **76,** 477–480.
Goldin, A., Goldberg, B., Ortega, L. G., and Schoenbach, E. B. 1949a. *Cancer* **2,** 857–862.
Goldin, A., Goldberg, B., Ortega, L. G., Fugmann, R., Faiman, F., and Schoenbach, E. B. 1949b. *Cancer* **2,** 865–876.
Goldin, A., Greenspan, E. M., and Schoenbach, E. B. 1950. *J. Natl. Cancer Inst.* **11,** 319–338.
Goldin, A., Greenspan, E. M., and Schoenbach, E. B. 1952. *Cancer* **5,** 153–160.
Goodman, L. S., and Gilman, A. 1941. The Pharmacological Basis of Therapeutics, 1st ed. The Macmillan Co., New York.
Goodman, L. S., Wintrobe, M. M., Dameshek, W., Goodman, M. J., Gilman, A., and McLennan, M. T. 1946. *J. Am. Med. Assoc.* **132,** 126–132.
Gordon, M., Siri, J. B., and Campbell, J. G. 1951. *Science* **113,** 61–64.
Graef, I., Karnofsky, D. A., Jager, B. V., Krichesky, B., and Smith, H. W. 1948. *Am. J. Pathol.* **24,** 1–47.
Graff, S., Engelman, M., Gillespie, H. B., and Graff, A. M. 1951. *Cancer Research* **11,** 388–392.
Gratia, A., and Linz, R. 1931. *Compt. rend. soc. biol.* **108,** 427–428.
Greenberg, D. M., and Schulman, M. P. 1947. *Science* **106,** 271–272.
Greene, H. S. N. 1941. *J. Exptl. Med.* **73,** 461–474.
Greene, H. S. N., and Lund, P. K. 1944. *Cancer Research* **4,** 352–363.
Greene, H. S. N., and Murphy, E. D. 1945. *Cancer Research* **5,** 269–282.
Greene, H. S. N. 1951. *Cancer Research* **11,** 899–911.
Greenspan, E. M., Leiter, J., and Shear, M. J. 1950. *J. Natl. Cancer Inst.* **10,** 1295–1333.
Greenspan, E. M., Colsky, J., Schoenbach, E. B., and Shear, M. J. 1952. *Cancer Research* **12,** 266–267.
Greenstein, J. P. 1947. Biochemistry of Cancer, 1st ed. Academic Press, New York.
Güthert, H. 1949. *Zentr. allgem. Path. u. path. Anat.* **85,** 160–162.
Gye, W. E., Ludford, R. J., and Barlow, H. 1943. *Brit. Med. J.* **2,** 65–67.
Haddow, A., and Robinson, A. M. 1937. *Proc. Roy. Soc. (London)* **B122,** 442–476.
Haddow, A., Watkinson, J. M., and Paterson, E. 1937. *Proc. Roy. Soc. (London)* **B122,** 477–507.

Haddow, A., and Sexton, W. A. 1946. *Nature* **157,** 500–503.
Haddow, A., Kon, G. A. R., and Ross, W. C. J. 1948a. *Nature* **162,** 824–825.
Haddow, A., Harris, R. J. C., Kon, G. A. R., and Roe, E. M. F. 1948b. *Phil. Trans. Roy. Soc. (London)* **A241,** 147–195.
Haddow, A. 1949. *Proc. 1st Natl. Cancer Conf. (Memphis)* 88–94.
Haddow, A., and Timmis, G. M. 1950. *Abstr. 5th Intern. Congr. Cancer (Paris)* 90.
Haddow, A., and Timmis, G. M. 1953. *Lancet* **264,** I, 207–208.
Haddow, A. Unpublished data.
Hahn, P. F., Skipper, H. E., Carothers, E. L., and Bernard, L. J. 1951. *Cancer* **4,** 634–636.
Hamilton, L. Unpublished data.
Hartwell, J. L., Shear, M. J., and Adams, J. R., Jr. 1943. *J. Natl. Cancer Inst.* **4,** 107–122.
Hartwell, J. L., and Shear, M. J. 1947. *Cancer Research* **7,** 716–717.
Hauschka, T., Saxe, L. H., Jr., and Blair, M. 1947. *J. Natl. Cancer Inst.* **7,** 189–197.
Hauschka, T., and Goodwin, M. B. 1948. *Science* **107,** 600–602.
Hegner, C. A. 1913. *Münch. med. Wochschr.* **60,** 2722–2724.
Heilman, F. R., and Kendall, E. C. 1944. *Endocrinology* **34,** 416–420.
Heiman, J., and Meisel, D. 1949a. *Cancer* **2,** 329–334.
Heiman, J., and Meisel, D. 1949b. *Cancer* **2,** 335–336.
Heiman, J., and Meisel, D. 1949c. *Cancer* **2,** 337–340.
Hendry, J. A., Homer, R. F., Rose, F. L., and Walpole, A. L. 1950. *Abstr. 5th Intern. Congr. Cancer (Paris)* 87–88.
Hendry, J. A., Rose, F. L., and Walpole, A. L. 1951a. *Brit. J. Pharmacol.* **6,** 201–234.
Hendry, J. A., Homer, R. F., Rose, F. L., and Walpole, A. L. 1951b. *Brit. J. Pharmacol.* **6,** 235–255.
Hendry, J. A., Homer, R. F., Rose, F. L., and Walpole, A. L. 1951c. *Brit. J. Pharmacol.* **6,** 357–410.
Hertz, R. O., and Tullner, W. W. 1949. *Science* **109,** 539.
Hesselbach, M. L., Fischer, C., and Burk, D. 1947. Approaches to Tumor Chemotherapy, pp. 196–199. *Am. Assoc. Advancement Sci.*, Washington, D.C.
Heston, W. E. 1949. *J. Natl. Cancer Inst.* **10,** 125–130.
Heston, W. E. 1950. *J. Natl. Cancer Inst.* **11,** 415–423.
Higgins, G. M., and Woods, K. A. 1949. *Proc. Staff Meetings Mayo Clinic* **24,** 238–242.
Higgins, G. M., Woods, K. A., and Bennett, W. A. 1950a. *Cancer Research* **10,** 203.
Higgins, G. M., and Bennett, W. A. 1952. *J. Natl. Cancer Inst.* **12,** 851–859.
Hirschberg, E., Kream, J., and Gellhorn, A. 1952. *Cancer Research* **12,** 524–528.
Hirschberg, E., Murray, M. R., Peterson, E. R., Kream, J., Schafranek, R., and Pool, J. L. 1953. *Cancer Research* **13,** 153–157.
Hitchings, G. H., Elion, G. B., VanderWerff, H., and Falco, E. A. 1948. *J. Biol. Chem.* **174,** 765–766.
Hoch-Ligeti, C., and Hsü, Y. T. 1953. *Science* **117,** 360–361.
Holly, F. W., Peel, E. W., Mozingo, R., and Folkers, K. 1950. *J. Am. Chem. Soc.* **72,** 5416–5418.
Hoster, H. A., Zanes, R. P., Jr., and von Hamm, E. 1949. *Cancer Research* **9,** 473–480.
Huggins, C. 1942. *Ann. Surg.* **115,** 1192–1200.
Huggins, C. 1946. *J. Am. Med. Assoc.* **131,** 576–581.
Huggins, C., and Bergenstal, D. M. 1952. *Cancer Research* **12,** 134–141.
Hutner, S. H., and Zahl, P. A. 1943. *Proc. Soc. Exptl. Biol. Med.* **52,** 364–368.
Ikawa, M., Koepfli, J. B., Mudd, S. G., and Niemann, C. 1952. *J. Natl. Cancer Inst.* **13,** 157–166.
Ingle, D. J., Prestrud, M. C., and Rice, K. L. 1950. *Endocrinology* **46,** 510–513.

Ingle, D. J., and Nezamis, J. E. 1951. *Endocrinology* **48,** 484–485.
Irons, W. G. 1946. *J. Dental Research* **25,** 180.
Irons, W. G., and Boyd, E. F. 1952. *Arizona Med.* **9,** 39–45.
Ivy, A. C. 1951. Krebiozen: An Agent for the Treatment of Malignant Tumors. Chicago (privately published).
Jacobson, L. O., Spurr, C. L., Barron, E. S. G., Smith, T., Lushbaugh, C., and Dick, G. F. 1946. *J. Am. Med. Assoc.* **132,** 263–271.
Jacquez, J. A., Barclay, R. K., and Stock, C. C. 1951. *Cancer Research* **11,** 259.
Jacquez, J. A., Barclay, R. K., and Stock, C. C. 1952. *J. Exptl. Med.* **96,** 499–512.
Jaffé, W. G. 1947. *Cancer Research* **7,** 107–113.
Jawetz, E., Gunnison, J. B., Speck, R. S., and Coleman, V. R. 1951a. *Arch. Internal Med.* **87,** 349–359.
Jawetz E., Gunnison, J. B., and Speck, R. S. 1951b. *Am. J. Med. Sci.* **222,** 404–412.
Jawetz, E., and Gunnison J. B. 1952. *Antibiotics & Chemotherapy* **2,** 243–248.
Jukes, T. H., and Stokstad, E. L. R. 1948. *Physiol. Revs.* **28,** 51–106.
Kahler, H., Shear, M. J., and Hartwell, J. L. 1943. *J. Natl. Cancer Inst.* **4,** 123–129.
Karnofsky, D. A., Burchenal, J. H., Ormsbee, R. A., Cornman, I., and Rhoads, C. P. 1947a. Approaches to Tumor Chemotherapy, pp. 293–305. Am. Assoc. Advancement Sci., Washington, D.C.
Karnofsky, D. A., Craver, L. F., Rhoads, C. P., and Abels, J. C. 1947b. Approaches to Tumor Chemotherapy, pp. 319–337. Am. Assoc. Advancement Sci., Washington, D.C.
Karnofsky, D. A. 1948. *New Engl. J. Med.* **239,** 226–305.
Karnofsky, D. A., Patterson, P. A., and Ridgway, L. P. 1949. *Proc. Soc. Exptl. Biol. Med.* **71,** 447–452.
Karnofsky, D. A. 1950. *Advances in Internal Med.* **4,** 1–75.
Karnofsky, D. A., and Burchenal, J. H. 1950. *Am. J. Med.* **8,** 767–788.
Karnofsky, D. A., Stock, C. C., and Rhoads, C. P. 1950. *Federation Proc.* **9,** 290.
Karnofsky, D. A., Burchenal, J. H., Armistead, G. C., Jr., Southam, C. M., Bernstein, J. L., Craver, L. F., and Rhoads, C. P. 1951. *Arch. Internal Med.* **87,** 477–516.
Karnofsky, D. A. 1952. *Ann. Rev. Med.* **3,** 283–306.
Keston, A. S., Ball, R. P., Frantz, V. K., and Palmer, W. W. 1942. *Science* **95,** 362–363.
Kidd, H. A. 1943. *Brit. Med. J.* **2,** 67–69.
Kidd, J. G. 1947. *Science* **105,** 511–513.
Kidder, G. W., and Dewey, V. C. 1949. *J. Biol. Chem.* **179,** 181–187.
Kidder, G. W., Dewey, V. C., Parks, R. E., Jr., and Woodside, G. L. 1949. *Science* **109,** 511–514.
Kidder, G. W., Dewey, V. C., Parks, R. E., Jr., and Woodside, G. L. 1951. *Cancer Research* **11,** 204–211.
King, L. S., and Sullivan, M. 1946. *Science* **104,** 244–245.
King, C. G., Bittner, J. J., Copeland, D. H., Engel, R. W., Kensler, C. J., Kidder, G. W., Mider, G. B., Miller, J. A., Morris, H. P., Robbins, W. J., Salmon, W. D., Tannenbaum, A., Tatum, E. L., White, F. R., White, J., and White, P. R. 1947. *Ann. N.Y. Acad. Sci.* **49,** 1–140.
Kingsley-Pillers, E. M., Burchenal, J. H., Eliel, L. P., and Pearson, O. H. 1952. *J. Am. Med. Assoc.* **148,** 987–994.
Kirschbaum, A., Judd, T., Lu, C. S., Engstrom, R. M., and Mixer, H. W. 1948. *Proc. Soc. Exptl. Biol. Med.* **68,** 377–380.
Klein, G. 1951a. The Production of Ascites Tumors in Mice and Their Use in Studies on Some Biological and Chemical Characteristics of Neoplastic Cells. Almquist & Wiksells Boktryckeri Ab, Uppsala.

Klein, G. 1951b. *Exptl. Cell Research* **2,** 518–573.
Klein, G., and Klein, E. 1951. *Cancer Research* **11,** 466–469.
Klopp, C. T., Alford T. C., Bateman, J., Berry, G. N., and Winship, T. 1950. *Ann. Surg.* **132,** 811–832.
Klyueva, N. G., and Roskin, G. 1946. *Vrachebnoe Delo.* **26,** 107; *Am. Rev. Soviet Med.* **4,** 127–129.
Koller, P. C. 1949. *Proc. 8th Intern. Congr. Genet.; Hereditas* **Suppl.** 320–324.
Koller, P. C., and Casarini, A. 1952. *Brit. J. Cancer* **6,** 173–185.
Koller, P. C. Unpublished data.
Koprowski, H., and Norton, T. W. 1950. *Cancer* **3,** 874–885.
Laccasagne, A. 1939. *Ergeb. Vitamin u. Hormonforsch.* **2,** 259–296.
Lancet Editorial. 1952. 263, 173.
Landauer, W. 1947. *Endocrinology* **41,** 489–493.
Landschutz, C., and Muller-Dethard, H. 1949. *Z. Krebsforsch.* **56,** 373–378.
Larson, C. D. 1947. *J. Natl. Cancer Inst.* **8,** 99–101.
Larson, C. D. 1948. *J. Natl. Cancer Inst.* **9,** 35–37.
Laszlo, D., and Leuchtenberger, C. 1943a. *Science* **97,** 515.
Laszlo, D., and Leuchtenberger, C. 1943b. *Cancer Research* **3,** 401–410.
Lathrop, A. E. C., and Loeb, L. 1916. *J. Cancer Research* **1,** 1–19.
Law, L. W. 1947. *Proc. Soc. Exptl. Biol. Med.* **66,** 158–161.
Law, L. W., and Spiers, R. 1947. *Proc. Soc. Exptl. Biol. Med.* **66,** 226–230.
Law, L. W., Dunn, T. B., Boyle, P. J., and Miller, J. H. 1949. *J. Natl. Cancer Inst.* **10,** 179–192.
Law, L. W. 1950. *Cancer Research* **10,** 186–190.
Law, L. W., and Boyle, P. J. 1950. *Proc. Soc. Exptl. Biol. Med.* **74,** 599–602.
Law, L. W. 1951. *J. Natl. Cancer Inst.* **11,** 849–865.
Law, L. W. 1952. *Cancer Research* **12,** 871–878.
Lees, T. W., and Lees, J. C. 1950. *Cancer* **3,** 580–600.
Lefevre, J. 1939. *Compt. rend.* **208,** 301–304.
Leiter, J., Downing, V., Hartwell, J. L., and Shear, M. J. 1950. *J. Natl. Cancer Inst.* **10,** 1273–1293.
Leiter, J., Downing, V., Hartwell, J. L., and Shear, M. J. 1952a. *J. Natl. Cancer Inst.* **13,** 365–378.
Leiter, J., Downing, V., Hartwell, J. L., and Shear, M. J. 1952b. *J. Natl. Cancer Inst.* **13,** 379–392.
Leiter, J., Hartwell, J. L., Kline, I., Nadkarni, M. V., and Shear, M. J. 1952c. *J. Natl. Cancer Inst.* **13,** 731–739.
Leiter, J., Hartwell, J. L., Ullyot, G. E., and Shear, M. J. 1953. *J. Natl. Cancer Inst.* **13,** 1201–1213.
Lettré, H. 1941. *Hoppe-Seyler's Z. physiol. Chem.* **268,** 59–76.
Lettré, H. 1950. *Z. Krebsforsch.* **57,** 1–13.
Lettré, H. 1951. *Angew. Chem.* **63,** 421–430.
Leuchtenberger, C., Lewisohn, R., Laszlo, D., and Leuchtenberger, R. 1944. *Proc. Soc. Exptl. Biol. Med.* **55,** 204–205.
Leuchtenberger, R., Leuchtenberger, C., Laszlo, D. and Lewisohn, R. 1945. *Science* **101,** 46.
Levaditi, C., and Nicolau, S. 1923. *Ann. inst. Pasteur* **37,** 443–447.
Levaditi, C., and Haber, F. 1937. *Rev. immunol.* **3,** 5–24.
Lewin, R., Spencer, H., Ekstein, D. M., Woidowsky, L., Stern, K. G., and Laszlo, D. 1952. *Cancer Research* **12,** 278–279.

Lewis, M. R. 1944. *Science* **100,** 314–315.
Lewis, M. R., Sloviter, H. A., and Goland, P. P. 1946. *Anat. Record* **95,** 89–96.
Lewis, M. R., and Goland, P. P. 1947. *Anat. Record* **99,** 369–378.
Lewis, M. R., Goland, P. P., and Sloviter, H. A. 1949. *Cancer Research* **9,** 736–740.
Lewis, M. R., and Crossley, M. L. 1950. *Arch. Biochem.* **26,** 319–320.
Lewisohn, R. 1938. *Surg. Gynecol. Obstet.* **66,** 563–576.
Lewisohn, R., Leuchtenberger, C., Leuchtenberger, R., and Keresztesy, J. C. 1946. *Science* **104,** 436–437.
Lewisohn, R. 1947. Approaches to Tumor Chemotherapy, pp. 139–147. Am. Assoc. Advancement Sci., Washington, D.C.
Lipschutz, A. 1950. Steroid Hormones and Tumors, 1st ed. Williams and Wilkins Co., Baltimore.
Lits, F. J., Kirschbaum, A., and Strong, L. C. 1938. *Am. J. Cancer* **34,** 196–213.
Little, P. A., Sampath, A., Paganelli, V., Locke, E., and SubbaRow, Y. 1948a. *Trans. N.Y. Acad. Sci.* **10,** 91–98.
Little, P. A., Oleson, J. J., and SubbaRow, Y. 1948b. *J. Lab. Clin. Med.* **33,** 1139–1143.
Little, P. A., Sampath, A., and SubbaRow, Y. 1948c. *J. Lab. Clin. Med.* **33,** 1144–1149.
Loeb, L. 1919. *J. Med. Research* **40,** 477–496.
Loefer, J. B. 1951. *Cancer Research* **11,** 481–484.
Loeser, A. A. 1938. *Brit. Med. J.* **II,** 319.
Loveless, A., and Revell, S. 1950. *Nature* **164,** 938–944.
Loveless, A., and Ross, W. C. J. 1950. *Nature* **166,** 1113–1114.
Loveless, A. 1951. *Nature* **167,** 338–342.
Ludford, R. J. 1945. *J. Natl. Cancer Inst.* **6,** 89–101.
Ludford, R. J. 1948. *Brit. J. Cancer* **2,** 75–86.
Lushbaugh, C. C., Green, J. W., Jr., and Storer, J. B. 1948. *J. Natl. Cancer Inst.* **8,** 201–207.
Malisoff, W. M. 1947. *Science* **106,** 591–594.
Meyer, J. R. 1945. *Arquiv. inst. biol. (São Paulo)* **16,** 307–314.
Meyer, J., and Weinmann, J. P. 1951. *Cancer Research* **11,** 914–917.
Meyer, L. M., Fink, H., Sawitsky, A., Rowen, M., and Ritz, N. D. 1949. *Am. J. Clin. Pathol.* **19,** 119–126.
Miller, F. R., Wearn, J. T., and Heinle, R. W. 1939. *Proc. Soc. Exptl. Biol. Med.* **41,** 479–480.
Miller, F. R., Herbut, P. A., and Jones, H. W. 1947. *Blood* **2,** 15–39.
Mitchell, J. S. 1948. *Brit. J. Cancer* **2,** 351–359.
Mitchell, J. H., Jr., Skipper, H. E., and Bennett, L. L., Jr. 1950. *Cancer Research* **10,** 647–649.
Montanez, G., Murphy, E. A., and Dunn, M. S. 1951. *Cancer Research* **11,** 834–838.
Moore, A. E. 1949a. *Cancer* **2,** 516–524.
Moore, A. E. 1949b. *Cancer* **2,** 525–534.
Moore, A. E., Stock, C. C., Sugiura, K., and Rhoads, C. P. 1949. *Proc. Soc. Exptl. Biol. Med.* **70,** 396–398.
Moore, A. E., and O'Connor, S. 1950. *Cancer* **3,** 886–890.
Moore, A. E. 1951a. *Acta Unio Intern. contra Cancrum* **7,** 278–281.
Moore, A. E. 1951b. *Cancer* **4,** 375–382.
Moore, A. E. 1951c. *Proc. Soc. Exptl. Biol. Med.* **76,** 749–754.
Moore, A. E., and Friend, C. 1951. *Proc. Soc. Exptl. Biol. Med.* **78,** 153–157.
Moore, A. E. 1952. *Ann. N.Y. Acad. Sci.* **54,** 945–952.
Morris, H. P., and Lippincott, S. W. 1941. *J. Natl. Cancer Inst.* **2,** 47–54.

Morris, H. P. 1947. Approaches to Tumor Chemotherapy, pp. 195–196. Am. Assoc. Advancement Sci., Washington, D.C.
Movitz, D., Saphir, O., and Strauss, A. A. 1949. *Cancer Research* **9**, 17–26.
Murphy, J. B. 1912. *J. Am. Med. Assoc.* **59**, 874–875.
Murphy, J. B. 1913. *J. Exptl. Med.* **17**, 482–493.
Murphy, J. B. 1914. *J. Am. Med. Assoc.* **62**, 1459.
Murphy, J. B., and Sturm, E. 1944. *Science* **99**, 303.
Murphy, J. B., and Sturm, E. 1947. *Cancer Research* **7**, 417–420.
Murphy, M. L. Unpublished data.
Nakahara, W. 1952. *J. Natl. Cancer Inst.* **12**, 960–962.
Nathanson, I. T. 1947. *Recent Progr. Hormone Research* **1**, 261–291.
Nauts, H. C., Swift, W. E., and Coley, B. L. 1946. *Cancer Research* **6**, 205–216.
Nauts, H. C., Fowler, G. A., and Bogatko, F. H. 1953. *Acta. Med. Scand.* Suppl. **276**, 145.
Nettleship, A., and Henshaw, P. S. 1943. *J. Natl. Cancer Inst.* **4**, 309–319.
Neukomm, S., Thompson, C. B., and Boissonaz, A. 1949. *Experientia* **5**, 239–241.
Niedner, K. 1941. *Z. Krebsforsch.* **51**, 159–163.
Nutini, L. G., Le Tang, D., and Biondo, F. X. 1952. *Acta Unio Intern. contra Cancrum* **7**, 794–800.
O'Flynn, C. P. 1950. *Med. J. Australia* **I**, 356–359.
Oleson, J. J., and Little, P. A. 1949. *Proc. Soc. Exptl. Biol. Med.* **71**, 226–227.
Ormsbee, R. A., Cornman, I., and Berger, R. E. 1947. *Proc. Soc. Exptl. Biol. Med.* **66**, 586–590.
Pack, G. T. 1950. *Arch. Dermatol. and Syphilol.* **62**, 694–695.
Parsons, L. D., Gulland, J. M., and Barker, G. R. 1947. *Symp. Soc. exptl. Biol.* **1**, 179–189.
Paterson, E., Haddow, A., Thomas, J. A., and Watkinson, J. M. 1946. *Lancet* **I**, 677–682.
Paterson, E., and Boland, J. 1951. *Brit. J. Cancer* **5**, 28–37.
Patt, D. I., Handler, A. H., and Lutz, B. R. 1952. *Cancer Research* **12**, 287.
Patti, J., and Moore, A. E. 1950. *Cancer Research* **10**, 674–678.
Patti, J., and Biesele, J. J. 1951. *Cancer Research* **11**, 540–542.
Pearson, O. H., Eliel, L. P., Rawson, R. W., Dobriner, K., and Rhoads, C. P. 1949. *Cancer* **2**, 943–945.
Personeus, G., Halliday, S. L., McKenzie, D., and Williams, J. H. 1952. *Proc. Soc. Exptl. Biol. Med.* **81**, 614–616.
Petering, H. G. 1952. *Physiol. Revs.* **32**, 197–213.
Philips, F. S., and Thiersch, J. B. 1949a. *Proc. Soc. Exptl. Biol. Med.* **72**, 401–408.
Philips, F. S., and Thiersch, J. B. 1949b. *J. Pharmacol. Exptl. Therap.* **95**, 303–311.
Philips, F. S., and Thiersch, J. B. 1950. *J. Pharmacol. Exptl. Therap.* **100**, 398–407.
Philips, F. S. 1950. *J. Pharmacol. Exptl. Therap.* **99**, 281–323.
Phillips, R., Karnofsky, D. A., Hamilton, L. D., and Nickson, J. J. *Am. J. Roentgenol. Radium Therapy.* In press.
Plummer, J. I. 1952. *Blood* Suppl. **7**, 181–182.
Pomerat, C. M. 1945. *Cancer Research* **5**, 724–728.
Potter, V. R. 1951. *Proc. Soc. Exptl. Biol. Med.* **76**, 41–46.
Pressman, D. 1949. *Cancer* **2**, 697–700.
Rawson, R. W., Skanse, B. N., Marinelli, L. D., and Fluharty, R. G. 1949. *Cancer* **2**, 279–292.
Reilly, H. C., and Stock, C. C. 1951. *Cancer Research* **11**, 366–369.
Reilly, H. C., Stock, C. C., Buckley, S. M., and Clarke, D. A. 1953. *Cancer Research* **13**, 684–687.

Reilly, H. C. 1953. *Cancer Research* **13,** 821–834.
Reimann, S. P., and Nishimura, E. T. 1949. *J. Michigan State Med. Soc.* **48,** 453-457
Rhoads, C. P., and Abels, J. C. 1943. *J. Am. Med. Assoc.* **121,** 1261–1263.
Rhoads, C. P. 1946. *J. Am. Med. Assoc.* **131,** 656–658.
Rhoads, C. P. 1949. *Bull. N.Y. Acad. Med.* **25,** 271–284.
Rhoads, C. P., Karnofsky, D. A., Burchenal, J. H., and Craver, L. F. 1950. *Trans. Assoc. Am. Physicians* **63,** 136–146.
Rhoads, C. P. 1951. *Science* **114,** 285–286.
Rickes, E. L., Brink, N. G., Koniuszy, F. R., Wood, T. R., and Folkers, K. 1948. *Science* **107,** 396–397.
Ridgway, L. P., and Karnofsky, D. A. 1952. *Ann. N.Y. Acad. Sci.* **55, II,** 203-215.
Rivers, T. M., and Pearce, L. 1925. *J. Exptl. Med.* **42,** 523–537.
Roblin, R. O., Jr., Lampen, J. O., English, J. P., Cole, Q. P., and Vaughn, J. R., Jr. 1945. *J. Am. Chem. Soc.* **67,** 290–294.
Roskin, G. 1946. *Cancer Research* **6,** 363–365.
Ross, W. C. J. 1950a. *J. Chem. Soc.* 752–753.
Ross, W. C. J. 1950b. *J. Chem. Soc.* 2257–2272.
Ross, W. C. J. 1950c. *J. Chem. Soc.* 815–818.
Ross, W. C. J. 1953. *Advances in Cancer Research* **1,** 397.
Sawitsky, A., Ritz, N. D., Jacobson, J., Meyer, L. M., Schwartz, S. O., Krim, N., Bock, G., and Brahin, C. M. 1952. *Cancer* **5,** 344–353.
Schoen, R. 1937. *Compt. rend. soc. biol.* **125,** 939–941.
Schoenbach, E. B., Goldin, A., Goldberg, B., and Ortega, L. G. 1949. *Cancer* **2,** 57–64.
Schoenbach, E. B., Colsky, J., and Greenspan, E. M. 1952. *Cancer* **5,** 1201–1220.
Shapiro, D. M., and Landing, B. H. 1948. *Science* **108,** 304–305.
Shapiro, D. M., Goldin, A., Landing, B. H., Bergner, A. D., Faiman, F., and Goldberg, B. 1949. *Cancer* **2,** 100–112.
Shapiro, D. M., Weiss, R., and Gellhorn, A. 1950. *Cancer* **3,** 896–902.
Shapiro, D. M., and Gellhorn, A. 1951. *Cancer Research* **11,** 35–41.
Shapiro, D. M. 1952. *Cancer Research* **12,** 713–715.
Shapiro, D. M., and Fugmann, R. A. 1952. *Proc. Soc. Exptl. Biol. Med.* **81,** 239–242.
Shapiro, D. M., Kream, J., and Dietrich, L. S. 1952. *Proc. Soc. Exptl. Biol. Med.* **81,** 616–619.
Sharpless, G. R., Davies, M. C., and Cox, H. R. 1950. *Proc. Soc. Exptl. Biol. Med.* **73,** 270–275.
Shear, M. J., and Andervont, H. B. 1936. *Proc. Soc. Exptl. Biol. Med.* **34,** 323–325.
Shear, M. J., and Turner, F. C. 1943. *J. Natl. Cancer Inst.* **4,** 81–97.
Shear, M. J., Hartwell, J. L., Peters, V. B., Dalton, A. J., Dunn, T. B., Hauschka, T., Rees, C. W., Diller, I. C., Beck, L. V., McConnell, J. R., Holloman, A. L., Oakey, R., and Reimann, S. P. 1947. Approaches to Tumor Chemotherapy, pp. 236–284. Am. Assoc. Advancement Sci., Washington, D.C.
Shimkin, M. B., and Bierman, H. R. 1949a. *Proc. Soc. Exptl. Biol. Med.* **71,** 250–252.
Shimkin, M. B., and Bierman, H. R. 1949b. *Radiology* **53,** 518–529.
Skipper, H. E., Bryan, C. E., Riser, W. H., Jr., Welty, M., and Stelzenmuller, A. 1948. *J. Natl. Cancer Inst.* **9,** 77–88.
Skipper, H. E. 1949. *Cancer* **2,** 475–479.
Skipper, H. E., and Bryan, C. E. 1949. *J. Natl. Cancer Inst.* **9,** 391–397.
Skipper, H. E. 1950. *Texas Repts. Biol. Med.* **4,** 543–550.
Skipper, H. E., Edwards, P. C., Bryan, C. E., Chapman, J. B., Bell, M., and Hutchison, O. S. 1950a. *Cancer* **3,** 348–353.

Skipper, H. E., Bennett, L. L., Jr., Edwards, P. C., Bryan, C. E., Hutchison, O. S., Chapman, J. B., and Bell, M. 1950b. *Cancer Research* **10,** 166–169.
Skipper, H. E., Mitchell, J. H., Jr., and Bennett, L. L., Jr. 1950c. *Cancer Research* **10,** 510–512.
Skipper, H. E., Chapman, J. B., and Bell, M. 1951a. *Cancer Research* **11,** 109–112.
Skipper, H. E., Bennett, L. L., Jr., Bryan, C. E., White, L., Jr., Newton, M. A., and Simpson, L. 1951b. *Cancer Research* **11,** 46–51.
Skipper, H. E., Mitchell, J. H., Jr., Bennett, L. L., Jr., Newton, M. A., Simpson, L., and Eidson, M. 1951c. *Cancer Research* **11,** 145–149.
Skipper, H. E., 1953. Unpublished data.
Sloviter, H. A. 1949. *Cancer Research* **9,** 677–680.
Sommers, S. C., Chute, R. N., and Warren, S. 1952a. *Cancer Research* **12,** 909–911.
Sommers, S. C., Sullivan, B. A., and Warren, S. 1952b. *Cancer Research* **12,** 915–917.
Southam, C. M., and Moore, A. E. 1951. *Am. J. Trop. Med.* **31,** 724–741.
Southam, C. M., Bronstein, B., and Webber, L. F. 1951. *Cancer Research* **11,** 669–675.
Southam, C. M., and Moore, A. E. 1952. *Cancer* **5,** 1025–1034.
Speck, R. S., Jawetz, E., and Gunnison, J. B. 1951. *Arch. Internal Med.* **88,** 168–174.
Stevens, C. D., Wagner, M. A., Quinlin, P. M., and Kock, A. M. 1952. *Cancer Research* **12,** 634–639.
Stock, C. C., and Rhoads, C. P. 1949. Evaluation of Chemotherapeutic Agents, pp. 181–190. Columbia University Press, New York.
Stock, C. C., Cavalieri, L. F., Hitchings, G. H., and Buckley, S. M. 1949a. *Proc. Soc. Exptl. Biol. Med.* **72,** 565–567.
Stock, C. C., Sugiura, K., and Rhoads, C. P. 1949b. *Acta Unio Intern. contra Cancrum* **6,** 550–554.
Stock, C. C. 1950. *Am. J. Med.* **8,** 658–674.
Stock, C. C., Biesele, J. J., Burchenal, J. H., Karnofsky, D. A., Moore, A. E., and Sugiura, K. 1950. *Ann. N.Y. Acad. Sci.* **52,** 1360–1378.
Stock, C. C., and Buckley, S. M. 1950. *Abstr. 5th Intern. Congr. Cancer (Paris)* 85.
Stock, C. C. 1951. First Symposium on Chemical-Biological Correlation, pp. 370–379. National Research Council, Washington, D.C.
Stock, C. C., Buckley, S. M., Sugiura, K., and Rhoads, C. P. 1951a. *Cancer Research* **11,** 432–436.
Stock, C. C., Karnofsky, D. A., and Sugiura, K. 1951b. Symposium on Steroids in Experimental and Clinical Practice, pp. 50–71. The Blakiston Co., Philadelphia.
Stock, C. C., Jacobson, W., and Williamson, M. 1952. *Proc. Soc. Exptl. Biol. Med.* **78,** 874–876.
Stock, C. C., Philips, F. S., Moore, A. E., Buckley, S. M., Clarke, D. A., Barclay, R. K., and Sugiura, K. 1953. *Cancer Research* **Suppl. 1,** 91–179.
Stock, C. C., and Reilly, H. C. Unpublished data.
Stock, C. C., *et al.* Unpublished data.
Stoerck, H. C. 1947. *J. Biol. Chem.* **171,** 437–438.
Stoerck, H. C., and Emerson, G. A. 1949. *Proc. Soc. Exptl. Biol. Med.* **70,** 703–704.
Strauss, R. 1946. *J. Immunol.* **54,** 151–154.
Sugiura, K. 1931. *Am. J. Cancer* **15,** (2), 707–724.
Sugiura, K. 1938. *Am. J. Cancer* **32,** 126–134.
Sugiura, K. 1947. Approaches to Tumor Chemotherapy, pp. 208–213. Am. Assoc. Advancement Sci., Washington, D.C.
Sugiura, K., Moore, A. E., and Stock, C. C. 1949. *Cancer* **2,** 491–502.
Sugiura, K., Hitchings, G. H., Cavalieri, L. F., and Stock, C. C. 1950a. *Cancer Research* **10,** 178–185.

Sugiura, K., Stock, C. C., Dobriner, K., and Rhoads, C. P. 1950b. *Cancer Research* **10,** 244–245.
Sugiura, K., and Stock, C. C. 1951. *Cancer Research* **11,** 284–285.
Sugiura, K., and Stock, C. C. 1952. *Cancer* **5,** 979–991.
Sugiura, K. 1953. *Proc. Am. Assoc. Cancer Research* **1,** 55.
Sugiura, K. Unpublished data.
Sullivan, R. D., Jones, R., Jr., Schnabel, T. G., Jr., and McC. Shorey, J. 1953a. *Cancer* **6,** 121–134.
Sullivan, R. D., Mescom, H., and Jones, R., Jr., 1953b. *Cancer* **6,** 288–293.
Sykes, M. P., Karnofsky, D. A., Philips, F. S., and Burchenal, J. H. 1953. *Cancer* **6,** 142–148.
Tannenbaum, A. 1947. *Ann. N.Y. Acad. Sci.* **49,** 5–18.
Tannenbaum, A., and Silverstone, H. 1949. *Cancer Research* **9,** 162–173.
Taylor, A., Thacker, J., and Pennington, D. 1942. *Science* **96,** 342–343.
Taylor, A., Hungate, R. E., and Taylor, D. R. 1943. *Cancer Research* **3,** 537–541.
Taylor, A., Carmichael, B. A., and Norris, B. S. 1948. *Cancer Research* **8,** 264–269.
Taylor, A. 1950. *Texas Repts. Biol. Med.* **8,** 227–237.
Taylor, A., Carmichael, N., McKenna, G. F., and Burlage, H. M. 1951. *Proc. Soc. Exptl. Biol. Med.* **77,** 841–843.
Templeton, W. G., and Sexton, W. A. 1945. *Nature* **156,** 630.
Thiersch, J. B., and Philips, F. S. 1949. *Proc. Soc. Exptl. Biol. Med.* **71,** 484–490.
Thiersch, J. B., and Stock, C. C. 1949. *Cancer* **2,** 863–864.
Thompson, R. L., Wilkin, M. L., Hitchings, G. H., Elion, G. B., Falco, E. A., and Russell, P. B. 1949. *Science* **110,** 454.
Timmis, G. M. 1951. *Abstr. 12th Intern. Congr. Pure Appl. Chem.* (*New York*) 334–335.
Toolan, H. W. 1951a. *Proc. Soc. Exptl. Biol. Med.* **77,** 572–578.
Toolan, H. W. 1951b. *Proc. Soc. Exptl. Biol. Med.* **78,** 540–543.
Toolan, H. W., and Moore, A. E. 1952. *Proc. Soc. Exptl. Biol. Med.* **79,** 697–702.
Toolan, H. W. 1952. Personal Communication.
Toolan, H. W. 1953. *Proc. Am. Assoc. Cancer Research* **1,** 56.
Truhaut, R. 1952. *In* Actualités Pharmacologiques, 5 ème série. Masson et Cie, Paris.
Turner, D. L., and Miller, F. R. 1947. Approaches to Tumor Chemotherapy, pp. 64–76. Am. Assoc. Advancement Sci., Washington, D.C.
Turner, J. C., and Mulliken, B. 1947. *Cancer Research* **7,** 774–778.
Turner, J. C., and Mulliken, B. 1950. *Cancer* **3,** 354–360.
Vrat, V. 1951a. *Permanente Foundation Oakland, Calif. Med. Bull.* **9,** 49–55.
Vrat, V. 1951b. *Permanente Foundation Oakland, Calif. Med. Bull.* **9,** 56–59.
Wade, N. J., Hodapp, E. L., and Musacchia, B. C. 1951. *Cancer Research* **11,** 287.
Walaszek, E. J., Kelsey, F. E., and Geiling, E. M. K. 1952. *Science* **116,** 225–227.
Walpole, A. L. 1951. *Brit. J. Pharmacol.* **6,** 135–143.
Weir, D. R., and Heinle, R. W. 1947. *Proc. Soc. Exptl. Biol. Med.* **66,** 268–270.
Welch, A. D., and Heinle, R. W. 1951. *Pharmacol. Revs.* **3,** 345–411.
West, P. M. 1949. *Proc. Soc. Exptl. Biol. Med.* **71,** 252–253.
White, J. W. 1893. *Ann. Surg.* **18,** 152–188.
Williams, J. L., and Walters, C. L. 1947. *Nature* **159,** 503–504.
Williams, J. H., McKenzie, D., Halliday, S., Personeus, G. R., Stevens, M. L., Sparks, S. J., Smith, S. G., Troy, W. P., Schurr, H. S., Gleason, H. R., James, E. R., Moser, L., Lydick, P., Landes, M. J., Eve, V., Stick, P., and Vincent, N. 1952. *Cancer Research* **12,** 310.
Williams, G. Z., and Williams, J. T. 1952. *Cancer Research* **12,** 309–310.
Wiswell, O. B. 1951. *Proc. Soc. Exptl. Biol. Med.* **76,** 588–589.

Woll, E. 1948. *Trans. N.Y. Acad. Sci.* **10,** 83–91.
Woodhouse, D. L. 1943. *Brit. Med. J.* **II,** 231–232.
Woolley, G. W. 1950a. *Proc. Soc. Exptl. Biol. Med.* **74,** 286–289.
Woolley, G. W. 1950b. *Trans. N.Y. Acad. Sci.* **13,** II, 64–66.
Woolley, G. W. 1950c. *Abstr. 5th Intern. Congr. Cancer* 37.
Woolley, G. W., and Peters, B. A. 1953. *Proc. Soc. Exptl. Biol. Med.* **82,** 286–287.
Woolley, D. W. 1952. *Abstr. 2nd Intern. Congr. Biochem. (Paris)* 486–487.
Woolley, D. W. 1953. *Proc. Natl. Acad. Sci.* **39,** 6–18.
Wright, J. C., Prigot, A., Wright, B. P., Weintraub, S., and Wright, L. T. 1951. *J. Natl. Med. Assoc.* **43,** 211–240.
Wright, B. P., Wright, J. C., Prigot, A., Wright, L. T., and Weintraub, S. 1952a. *Harlem Hosp. Bull.* **4,** 151–163.
Wright, J. C., Prigot, A., Wright, L. T., and Arons, I. 1952b. *Arch. Internal Med.* **89,** 387–404.
Wright, L. T., Wright, J. C., Prigot, A., and Weintraub, S. 1950. *J. Natl. Med. Assoc.* **42,** 343–351.
Yoshida, T. 1952. *J. Natl. Cancer Inst.* **12,** 947–962.
Yu, S. T. 1947. *Abstr. 4th Intern. Congr. Cancer* 105.
Zahl, P. A., Hutner, S. H., Spitz, S., Sugiura, K., and Cooper, F. S. 1942. *Am. J. Hyg.* **36,** 224–242.
Zahl, P. A., Starr, M. P., and Hutner, S. H. 1945. *Am. J. Hyg.* **41,** 41–56.

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