Progress in Toxicology

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Volume 2

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I

INTRODUCTION

THE STATE OF THE ART

The recent seventies have not been good times for toxicologists. Public confidence in us is shaken, mostly as a consequence of an unhappy sequence of spectacular failures: we have been surprised by a tragic transplacental carcinogenic effect of diethylstilbestrol, an old and widely prescribed synthetic estrogen. We are confronted with a small epidemic of an unusual liver tumor, angiosarcoma, induced by a simple chemical, vinylchloride, whose insiduous toxic potential we failed to recognize for years. Our clinical colleagues had to tell us about the ulcerogenic effect of as simple a substance as potassium chloride. We also just learned of an unusual toxic reaction, pseudolupus, developing in people taking a popular fixed-combination drug, Venocuran[R]. Admittedly, we could not have predicted this syndrome, even if we had considered the possibility.

We are still unable (to continue this self-flagellation) to explain the relationship between the appetite suppressant aminorex and the suddenly increased occurrence of a lung disease posing as primary pulmonary hypertension. And worse yet, we have no way to evaluate a new derivative of this class, and to predict whether or not it, too, will produce the deadly condition. We still, to my knowledge, have no plausible explanation why and how thalidomide caused phocomelia. Of course, we do diligently a set of teratological experiments with each new drug; we follow a protocol which has the blessing of all governmental regulatory agencies. But we really cannot guarantee that all possibilities for another teratogenic catastrophy have been eliminated.

We meet more and more clinicians who worry a great deal about potential induction of liver adenomas and peliosis hepatis possibly related to prolonged treatment with certain sex hormones. But we seem to be unable, with our experimental techniques, to provide guidance for further study of the urgent problem. (Don't be embarrassed if you have never heard about this strange disease called peliosis hepatis, I, too, had to search for it in several pathology books).

When you are down you suddenly realize that your true friends are few and far between. Your critics who used to be helpful and discrete

become vociferous and nasty. Toxicology, they say, is really too serious a matter to be left to the toxicologists. They call for new approaches, new expert committees, and new advisory boards. It is amazing to see how many people who never in their lives conducted a toxicity experiment are now telling us how it should be done. We suddenly have a surprising number of experts who seem to know all about mutagenicity and carcinogenicity, and who force upon us their favorite tests and procedures. These experts also go witch-hunting. Among their most recent trophies they will show you cyclamate, LSD, a spray glue, Violet No. 1, and practolol.

All this illustrates that toxicology today is in a turmoil. Our neatly elaborated testing guidelines, and that should now be clear to everyone, have not solved all the problems. And there are no simple rules which will do it.

How then should we react to this crisis situation? First of all, let us agree that toxicology must develop scientifically, that it must gobble up all new knowledge generated by other biological disciplines. Let us listen to the advice of eminent scientists as well as our most vehement critics. Write to the National Academy of Sciences and ask for a copy of "How Safe is Safe? The design and Policy on Drugs and Food Additives" (1974). This book will give you food for thought, more than almost anyone can bear.

But let us not, however, discard all we have learned and accumulated in the past. Do not rush into new approaches just because they are new and are riding the popularity wave. And please do make an effort to always gauge the magnitude of every toxicological problem before wasting your precious resources.

THE TRIAL

It had to happen sooner or later. And it happened in Summer and early Fall of 1974: Drug Research and Toxicology were brought to justice in a public and well publicized trial. The occasion was a series of Senate hearings of the Subcommittee on Health of the Committee on Labor and Public Welfare. Presiding over the spectacle and acting as chief prosecutor and judge was the Honorable Senator from Massachusetts, Mr. Edward M. Kennedy.

Many scientists, particularly those enjoying the well sheltered life in academic institutions, are generally not interested in such hearings. What is said down in Washington D.C. is not published in the journals they read, and most of the things discussed, they believe, neither helps nor disturbs their own work. True, time is wasted in such hearings with trivial matters. At this occasion there was much talk about internal squabbles in certain government offices: who did and who did not receive copies of memoranda, who was not invited to a conference while he thought he should have been, who went to dinner or for drinks together and who was or wasn't asked to join, etc. These things, of course, will remain buried for ever in the Congressional Records.

But there were also more substantial matters discussed, and they did not, as Senator Javits assured the witnesses, "fall on deaf ears at all." "We are going to introduce legislation," was a recurring theme used by Mr. Kennedy. And that, my friends, should not fall on deaf ears in the scientific community. Just read the transcript of the proceedings of August 15, 1974 and you will be amazed what one of the witnesses said: "I cannot stress too strongly that I think that publications of investigations should be withheld until FDA review to prevent widespread use of the drug and then the FDA would have to curtail or supervise it more widely." And a few minutes later, the same witness reiterated: "... and I think we ought, in order tc prevent unsupervised use of drugs for non-approved uses that pre-approval publication must be prohibited. You have to get approval for publication of the results of any investigations on drugs for non-approved uses." With these words, Dr. Julia Apter, a professor of surgery in Chicago, advocated censorship of research results. Can we let such statements stand unchallenged, hoping they will be forgotten like the talk about the internal quarrels in FDA offices and expert committees? Or should we rate them as another threat, however small, to our freedom of scientific research? Whatever you decide, we must take such utterances by a distinguished member of the medical profession as a symptom of what is happening in drug research. And as such we cannot dismiss them lightly.

Another sign of the changing climate I mentioned already: it is the ever expanding circle of people getting involved in drug research. Making drugs, investigating them in animals, volunteers, and patients, putting them through registration procedures, and presenting them to the physicians are not any more the privilege of a rather small group of professionals. Large bureaucratic organizations in governmental agencies acting autonomously in every country, international agencies such as the World Health Organization, professional societies, medical and non-medical, powerful lay organizations such as consumer groups and the fighters for human rights, a clean environment, and animal welfare, the news media, legislators, and lawyers specializing in the malpractice business have learned to gain access to the scientific data and to subject them to their own interpretation. That leaves the professionals who generate the research results in a very precarious situation. They are forced to conduct the studies according to guidelines and practices which some of these critiques dislike or condemn. They often find themselves caught in a struggle between advocates of different statistical approaches. Even Senator Kennedy got a taste of it, when, after a lengthy discussion with a statistician, he desperately asked: "I thought that in the science of mathematics, you could get some definitive answers if you were looking at the same material. How can you reach a different conclusion by looking at the same statistical information?" (September 25, 1974).

One often calls in question the researchers' ability to evaluate their own results. Even the accuracy of their summaries is questioned as demonstrated by this verbal exchange at the hearing of August 15, 1974: Dr. Knox: "I find the sponsor's summary is not always what I would like, that it does not contain an analysis I would agree with." Senator Kennedy: "How about the rest of the panel? Do you agree with that as well, the importance of raw data?" Dr. Bryant: "Yes." Senator Kennedy: "Would the record show who agrees with that?" (Show of hands). End of quote.

To belabor this point further let me mention that the Committee, in Senator Kennedy's words, "has been interested in the area of adverse drug reaction." To this important topic it questioned one man, a physician whose training, before going into private practice, consisted of an internship in (of all places) a homeopathic general hospital. And the group who testified (on September 27, 1974) about two other most important subjects, the amount of animal testing necessary before exposing people to a new drug, and the clinical follow-up of research subjects, treated with a drug which, in subsequent experiments, is found to cause toxic effects in animals, consisted of a Mr. R. Nader who is not scientifically trained in medicine or pharmacology, and two lawyers. With this I do not mean to imply that these witnesses should not have been permitted to say their piece. All I want to point out is that those professionals who are actually doing the adverse reaction monitoring in hospitals and clinics day in and day out, and those who are charged with the responsibility of preparing new drugs for clinical trials should at least be given equal time. If consumer groups want to participate in scientific discussions on drug development and safety (and I strongly believe that they should) it would make life for all of us much easier, if they would agree to delegate someone soundly trained in the biomedical sciences.

In Senate hearings a layman asks the scientific questions and the scientist, testifying under oath, must answer them truthfully and in a language which is understood by the lay-public. To say the truth and nothing but the truth about scientific matters is difficult in itself. But to do it in a language which is understood by a lay-public is even harder. Moreover, at such hearings the scientist witness has no access to his papers and reference books, worse yet, he cannot show the slides which he normally happens to have in his coat pocket and which are his preferred tool in communicating with others. He gets increasingly disturbed because every word he mutters is taken down by a stonefaced stenographer, and he gets no comfort from the spectators among which he recognizes many newsmen lurking for headline-producing tidbits. Such a set-up is, of course, not suitable for a useful discussion of difficult scientific problems.

Why then are such hearings organized? You get different answers to this question depending on whom you ask. I tend to look at them as a somewhat primitive device to get problems which are stuck at many levels moving again. They are almost like the old doctors' attempts to influence a chronic illness by doing something drastic, to give a purge, perhaps, an injection of a pyrogen or even an electroshock. This does not cure the patient, of course, but it will often upset him enough so that he is now willing to accept more submissively the regimen which may ultimately bring about his recovery.

Let us, therefore, look once more at the transcript of the Senate hearings, but this time, not from the viewpoint of the wounded martyr. Let us rather act as cool recorders of the things several concerned colleagues and laymen believe are important shortcomings in today's research procedure on new drugs.

There certainly was no satisfaction among most of the witnesses with the way we do our preclinical animal toxicity studies. Some felt that we were just plain doing a poor job, others were more lenient and said that we were conducting our studies according to outdated guidelines.

Apparently the idea that insufficient guidelines are to blame for our failings has taken hold in several places. Large committees are at work in many countries to revise them once more. And the fruits of their deliberations are appearing just about now.

There were strong arguments for the concept that the preclinical safety evaluation should be completed before any human beings are exposed to a new drug. This postulate is sure to be received favorably by all those who are not personally involved in the process of drug development. But before putting it down as a binding requirement they should consider well the wise words of an experienced professional, Dr. M. Weiner, who told Mr. Kennedy on August 16, 1974: "Preclinical safety studies, including studies in animals, are never completed. We are still doing experiments and learning important new facts about drugs which have already proved useful over a period of hundreds of years. At each step in the drug research process judgments must be made as to how much and what kind of human exposure to a drug is justified by how much and what kind of preclinical testing."

One of the major worries of several witnesses was the thought that human subjects might be exposed to a new drug which, in subsequent animal tests, proves to be carcinogenic. In their opinion this threat justifies a regulation according to which all clinical testing of a new drug must be held in abeyance until carcinogenicity experiments are completed. Since this request is the main postulate of a petition of Mr. Nader's Public Citizens Health Research Group we are sure to witness a lively discussion about it in the months to come.

Intimately related to these requests are the worries of several witnesses about the follow-up of patients exposed to investigational drugs which, in subsequent animal tests, prove to cause toxic effects. How should patients and their physicians be informed? How can patients be monitored and how long should the follow-up continue?

The concern of the witnesses was generated by the recent discovery of unexpected toxic effects of a few drugs at the time when they were already in clinical trials. In all cases the clinical studies were immediately discontinued. But a state of generalized perplexity reigned about the question of what should now be done with the people who had already received the drugs. The difficulty of monitoring people for months, or, as in the case of carcinogenic effects, for many years, became obvious. In order to deal with similar occurrences in the future, the scientific community must accept the obligation of establishing reasonable principles for the follow-up of patients subjected to dangerous chemicals. But we must also make sure that the animal experiments which will set such a clinical monitoring program in motion are sound and really justify the enormous effort involved. Moreover, investigational drugs should not be treated differently from those already on the market. I say this because, (and the concerned witnesses were probably not aware of it), "disturbing" toxicological evidence can easily be produced in imaginative animal experiments with just about every drug. A short time in the library will be enough to concoct a horror story of all the potentially dangerous things some of our popular drugs can do if the experimental game in animals is played right (Zbinden, 1966). What we need then is a policy telling us what kind of animal data requires initiation of a patient follow-up. And for each type of toxicological problem our clinical colleagues must tell us which diagnostic procedures must be used.

In the discussion of patient follow-up the hearing briefly reached a considerable scientific niveau when the question of dose-effect relationship of chemical carcinogens was discussed. This problem becomes particularly sticky when we deal with drugs, which turn out to have some tumor-inducing effect in perhaps just one animal species or strain or only at excessively high doses. When should a patient who has been in contact with such a carcinogen be included in a life-long cancer-detection program? Can we define the risk from carcinogenic drugs, perhaps on a scale which is based on our experience with other proven carcinogenic influences in our environment, e.g. tobacco smoke, pollutants, myocotoxins, UV light, and x-ray exposure? There were strong opinions voiced about these matters during the hearing. Toxicologists are, therefore, well advised to put the problem high up on their priority list.

Other important points brought up by the witnesses included safety testing of drugs for pediatric use, the problem of patient injuries due to malprescribing and overprescribing, and the effect of the extreme concern about drug safety on the process of new drug discovery. All these problems certainly deserve our attention in the future.

Late in the hearings, on September 27, 1974, the discussion finally turned to what appears to be a most critical problem in drug research and development today. It is the performance of pharmacology in the area of drug discovery. As Dr. J.A. Oates of Nashville, Tenn. testified: "If one fails to discover a drug, this loss is absolute." When I see how many diseases are still unconquered and how many patients must die because of a lack of effective medication, I wonder whether our preoccupation with drug safety has not encroached upon the more important process of drug discovery. However, a superficial scan of over 800 pharmacology papers presented at the 1974 Federation Meeting in Atlantic City, N.J. (Table 1) does not support this suspicion. There were, indeed, rather few new drugs discussed by the American pharmacologists.

TABLE 1

Subjects of over 800 Papers Read by Pharmacologists at the 1974 Federation Meeting in Atlantic City, N.J.

Subject	%
Physiological studies incl. effects of endogenous substances	36.6 %
Pharmacological studies involving old drugs and non-drugs	32.8 %
Drug metabolism and pharmacokinetics	17.6 %
Pharmacological studies with new drugs	8.0 %
Toxicological studies with old drugs and non-drugs	4.5 %
Toxicological studies with new drugs	0.5 %

However, according to this survey, their interest does not seem to be diverted by toxicological preoccupations, but rather by their intense interest in physiological regulatory mechanisms and the desire to learn more about the pharmacological properties and the metabolism of old drugs. This indicates that modern pharmacology puts more emphasis on basic science than on new drug development. I don't want to speculate whether this is good or bad for the progress of medicine, but I also believe that it does not do too much to bring us the new drugs which are urgently required. Perhaps the process of drug discovery is also in need of some vigorous senatorial probing.

II

FORMAL TOXICOLOGY

THE ROLE OF PATHOLOGY IN TOXICITY TESTING

What's so Special about Toxicological Pathologists?

Sitting in judgment of their colleagues' mistakes, anatomical pathologists have always enjoyed a privileged status in medicine. Their predominant position in clinical diagnostics, however, is progressively eroded by usurpers using advanced biochemistry, sophisticated electronics, and immunological tricks. But in drug toxicology much of the old magic of the pathologist is still preserved. He is the only one permitted to use subjective judgments, the only one to coin his own technical terms. I can label the mammary nodules induced in beagle bitches with oral contraceptives as "ductal carcinoma in situ" and scare you know what out of everybody. I can call them "cystic fibromatous hyperplasias" and make it sound innocent; or I may not want to commit myself either way and name the change a "mammary hyperplasia with squamous metaplasia and a certain potential for malignant (carcinomatous or sarcomatous) degeneration."

In human surgical pathology great efforts are made to describe morphological lesions in detail and to establish criteria for diagnosis. This has not entirely eliminated chaos, but reasonable communication in a language understood by all is possible. Young pathologists are taught to comply with the rules of their elders. Most technical terms we use stand for well-defined disease processes to which only a few are added every year. The possibilities for drug-induced organ changes, however, are unlimited. Innumerable new lesions are generated continuously in toxicity tests all over the world, most of them are never published in the scientific literature. That means that only a small fraction of the total knowledge is known to any one pathologist. Thus, there _is_ something special about toxicological pathologists; it is their unique set of personal experiences, based on the tissue changes they have seen and the microscopic pictures they have stored in their memory. To communicate with colleagues, no generally accepted glossary is available. Pathologists trained in medical diagnostics continue to use their technical language which was developed to describe human diseases and degenerative processes. But drug-induced lesions, although they look

like changes typical for diseases, are often something completely different. The histopathological diagnosis of acute hepatitis in man points to a well-known condition and carries with it certain prognostic implications. The same histopathological change in a liver of a drug-poisoned mouse has a completely different origin, its further development cannot be predicted for lack of previous experience, and its significance as a toxic manifestation can only be speculated upon based on one's previous experience with related drugs and similar histopathological changes.

Pathological Studies in Subacute and Chronic Toxicity Tests

The experimental design of toxicity studies has been described in the first volume of Special Topics (ST I, 4-17). Briefly, groups of animals are treated for a certain period of time with at least 3 doses of the test compound. The high dose must cause some signs of toxicity, the low dose is a small multiple (2 to 10 x) of the projected therapeutic dose, and the intermediate level, well, this is the dose which fits in between the two others. An untreated group of at least equal size serves as control. At the end of the treatment the pathologist takes over and sees to it that the animals are killed, autopsied, and subjected to histopathological studies.

There is some disagreement as to the number of tissues to be processed for histopathology. Some pathologists examine what they call the major organs of all animals and take additional tissues only from the high dose group and part of the controls. Others follow the modern trend and look at as many tissues as possible from all animals including the controls. Those who have done this can truly claim that they have experienced a magnificent manifestation of the law of diminishing return. They have looked at hundreds of normal tissues, a boring experience bearable only if a generous renumeration is assured.

For many organs and tissues mass-produced paraffin sections and hematoxylin-eosin staining are sufficient. Frozen sections stained for fats are indispensable for liver, kidney, and adrenal glands, and probably also for spleen and heart. Other staining methods should be kept in reserve for special cases. For fixation I prefer Bouin's solution for the paraffin embedded material and 4% buffered formalin for frozen sections. Many other concoctions give equally good results. The main mistakes in preparing the tissues for histopathology are: failure to rinse off excess blood in isotonic saline before putting the specimens into the fixative, fixation of large pieces of tissue, squeezing the tissues and, worst of all, permitting the tissue specimens to adhere to the inside of the bottles which leads to irregular penetration of the fixative. The resulting artifacts may not fool, but will certainly annoy, the pathologist when he reviews the slides.

For practical purposes a reasonable compromise between an all encompassing evaluation of all possible tissues and a superficial examination is called for. In Table 2 I have therefore assembled a list of tissues and ranged them according to the frequency by which morphological changes are likely to occur. Under first priority those tissues which should be examined in all animals are listed. Tissues included under second priority might only be processed for the high

TABLE 2

Organs and Tissues to be Examined in Routine Toxicity Tests

First Priority: All animals including controls

Liver (f) [1]	Epididymis
Kidney (f)	Lung
Adrenal glands (f)	Bone marrow
Heart (left ventricle) (f)	Mesenterial lymph node
Spleen (f)	All organs showing gross changes of shape, weight, color, or structure
Thymus	
Testis	

Second Priority: High dose animals and part of controls. Medium and low dose animals only when significant changes observed in high dose group

Thyroid	Prostata
Parathyroid	Coagulating gland
Pituitary gland	Tonsils
Salivary glands	Brain
Stomach	Spinal cord
Duodenum	Eye
Small intestine	Optic nerve
Large intestine	Peripheral nerve
Pancreas	Urinary bladder
Ovary	Skin
Uterus	Mammary gland
Cervix uteri	Bone-cartilage
Vagina	Skeletal muscle
Seminal vesicles	Gall bladder

Third Priority: Organs and tissues not examined in routine experiments, unless indicated by clinical observations or motivated by scientific interest

Heart valves	Oviduct
Purkinje fibres	Vulva
Aorta and other blood vessels	Penis
Thoracic duct	Paraurethral and preputial glands
Tongue	Pineal gland
Teeth	Spinal ganglions and roots
Lips	Sympathetic ganglions and trunk
Gingivae	Nerve fibre endings
Hard palate	Meninges
Nasopharynx	External ear
Larynx	Middle ear
Trachea	Inner ear
Esophagus	Olfactory organ
Vermiform appendix	Subcutaneous lymph nodes
Rectum	Tendons
Anus	Adipose tissue
Ureter	Intervertebral disc
Urethra	Synovial membrane
	Lacrimal glands

1) (f) frozen sections stained for fat obligatory

dose group and part of the controls. Should any important change be observed, the tissues of the low- and intermediate-dose animals would also have to be examined. The third priority includes tissues which are rarely affected and do not need to be examined histopathologically unless indicated by clinical observation or motivated by a special interest of the pathologist.

For certain organs routine histopathology yields very little useful information unless special precautions are taken. This is particularly true for brain, spinal cord, peripheral nerve, spinal ganglions and roots, and the eye. A careful neuropharmacological and ophthalmoscopic control of the living animals is therefore most important in order to preselect those for which the very elaborate effort necessary for the proper histopathological evaluation of neural structures and the eye are indicated. (Alder and Zbinden, 1973). The urinary bladder of small animals must be filled with the fixative so that the mucosa is well expanded. Otherwise, detection of early changes is just about impossible. Specimens of skin and stomach mucosa should be pinned to a piece of cork to remain uncrumpled during fixation. Testicles should be fixed intact for about 4 hours. Only then should they be cut into 3 to 5 mm thick slices and put back in the fixative. Filling the lungs with fixative through the bronchi is occasionally called for but is not necessary as a routine procedure. Perfusion of the whole animal with saline followed by fixative is not recommended as a routine procedure. One more thing, the kidney should be placed in the fixative quite rapidly since active transport of various materials continues after death and leads to morphological changes of the tubular epithelium (Longley and Burstone, 1963).

Since we are talking trivials, I might add a few words about the best methods of sacrifice. This is not quite as unimportant as one may think. Pfeiffer and Muller (1967) have reviewed the subject and have done some experiments. For rats they recommend inhalation of ether or chloroform or i.p. injection of 500 mg/kg pentobarbital. In some laboratories asphyxia with city gas is practiced. Fortunately, the replacement of the CO-containing poison by natural gas will set an end to this unpleasant method of execution. In my laboratory the order is to use chloroform for all small rodents; in disobedience of it, everybody uses ether which must have some advantage unknown to me. To kill rabbits somebody once invented the method of i.v. injection of air. Don't do it! I.V. pentobarbital is the method to use. Dogs and monkeys may be killed by exsanguination in deep barbiturate anesthesia.

Toxicological Information which Is and Could Be Provided by Pathological Studies

The cost in time and money to conduct a routine pathological work-up of a toxicological experiment is considerable, and so is its informational value. Non-pathologists may think that histopathological studies would run a poor second when their performance is compared to other methods of investigation, such as clinical chemistry. That this is not necessarily true is proven by a number of studies of which a slightly biased selection shall be mentioned.

In rats, for example, the histopathologist was able to detect carbon tetrachloride-induced liver changes after oral administration of the minute amount of 0.0125 ml/kg. Function tests became positive only with increasing doses in the following decreasing order of sensivity:

1. Serum sorbitol dehydrogenase, serum urea
2. Isocitrate dehydrogenase, fructose-1-phosphate aldolase, SGPT, SGOT
3. Alcohol dehydrogenase, 6-phosphogluconate dehydrogenase, lactic and malic dehydrogenases (Korsrud et al., 1972)

The 2 most popular tests, SGOT and SGPT, also gave disappointing, i.e., negative, results in the evaluation of a mildly hepatotoxic choleretic agent, α-methyl-α-(2-morpholinoethyl)-1-naphthylacetic acid hydrochloride (DA 1627). In this case the simple determination of liver weight, another of the sometimes disdained pathologist's tools, was an even more sensitive indicator of hepatotoxicity than histopathology (Bianchi et al., 1968). Similar results were reported in dogs where a variety of mild or focal lesions such as hydropic vacuolization and fatty change of liver cells, inflammatory infiltration, siderosis of the Kupffer cells, and single cell necrosis were not accompanied by alterations of liver function tests (Hallesy and Benitz, 1963). One wonders why the technique of serial liver biopsies has not gained more popularity in drug toxicology.

To underline the superior performance of histopathology, toxicologists love to refer to the paper of Sharrat and Frazer (1963) which evaluates the usefulness of various methods in demonstrating kidney changes. Only histopathology recognized all of 15 acute or chronic glomerular or tubular injuries. Of the clinical laboratory procedures used to evaluate tubular damage, the best results were obtained with the microscopic examination of the urinary sediment, another morphological method. The other tests listed in decreasing order of reliability were:

1. Concentration test, serum urea
2. Phenol red test
3. Albuminuria and p-aminohippuric acid clearance
4. Dilution test (volume)
5. Dilution test (specific gravity)

Such studies have, of course, delighted and reassured the pathologists, but they also bred complacency. As a matter of fact, pathological studies as conventionally performed in toxicity testing do not provide as much information as they easily could. A moment of self-examination will make this evident.

A routine autopsy and a histopathological work-up of tissues of animals sacrified at the end of a toxicological test gives excellent information on the morphology of those drug-induced lesions which were present at this particular point in time. Moreover, the investigation

will also establish the presence or absence of a dose-effect relationship for some of the lesions.

Now consider what the pathologist's work does not provide: It gives no information about early lesions which disappear on continued treatment, but believe me, there are many of those, particularly in the very first days of a toxicity experiment. On further treatment the animals often adapt to the persistent chemical insult, be it by activation of drug metabolizing enzymes (ST I, 28-33) or by some other means. Pathologists who do not look at tissues from animals sacrificed at various stages of the experiment are like those who guess the beginning and the end of a 2 hour movie by glancing at a few frames. In many cases, a reasonable extrapolation is possible. But even some of today's movies and certainly many of today's new drugs are full of surprises which one must try to catch as they occur. The discretionary toxicity study as described earler (ST I, 10) is therefore again recommended.

By examining the animals at the end of an experiment, we miss all information about the sequence in which the lesions appear. If I am confronted with an animal which, after 3 months of exposure to drug A, has liver necrosis, brain edema, tubular nephrosis, atrophy of testicles, and bone marrow hypoplasia, how am I going to know what was first and what was only a consequence of one of the other drug-induced malfunctions? And if I investigate the related drug B which causes the same changes at the end of a 3 months experiment, might it not be relevant to know whether or not some or all of these changes required fewer or more daily treatments to produce the same changes as seen with drug A?

If I sacrifice all animals at the end of treatment, how am I going to know whether or not the lesions are reversible? Some pathologists seem to have a sixth sense for this when they write that a certain drug-induced lesion is "probably reversible." Those lacking this special intellectual capability can do nothing but to incorporate in their toxicity experiments special animal groups which are sacrificed 1 to 3 months after withdrawal of the drug. They will note then that some drug-induced lesions have disappeared, but others remain and some even get worse; and that is certainly relevant information which will carry more weight with clinical pharmacologists than any educated guess of even a famous pathologist.

Another important aspect most likely to be missed if tissues are examined at one point in time only is the development of morphological lesions. This is particularly well demonstrated with drugs causing damage to the testicle. After several months of treatment there is usually severe atrophy of the seminiferous tubules with perhaps a few spermatogonia left between twisted Sertoli cells. Tubules contain cellular debris, and there is interstitial edema and hypertrophy of the Leydig cells. But with some drugs this end point is reached following a rapidly developing inhibition of sperm maturation. With others maturation processes do not seem to be affected, but the damage is limited to one or several germinal cell types or directed towards the mitotic processes. It is evident that such observations on the morphogenesis are of great importance for the understanding and evaluation of drug-induced lesions.

At the beginning of this discussion I indicated that pathological examinations often permit the establishment of a dose-effect relationship. This is a most important parameter in toxicology, helpful in

distinguishing specific, drug-induced lesions from non-specific tissue changes occurring as a consequence of such extraneous factors as spontaneous disease, malnutrition, and degeneration related to old age or genetic influences. A dose-effect relationship can express itself by the severity of a certain change or by its relative incidence within the various treatment groups. In my experience it often manifests itself much better by the time the drug-induced changes appear. This becomes particularly useful when the lesion is present with equal frequency and severity at all dose levels or when it also occurs in the untreated control groups. The latter is often a serious complicating factor in carcinogenicity tests where establishment of a dose-effect relationship is so terribly important.

Acquisition and Handling of Pathological Data in Toxicology

By this time most non-pathologists have probably turned to more exciting reading, and I feel free to discuss additional technical matters. First, a word about the question of whether histopathological slides should be submitted under code or read with full knowledge of the specific treatment each animal has received. The purists' conviction is that unlabeled slides should be sent to the pathologist to ensure that the integrity of the control group is maintained during pathological evaluation (Fears and Schneiderman, 1974). As usual, the purists have all the arguments of good science on their side. The blind technique certainly reduces the investigator's bias, protects those reading the slides from charges of bias, and increases the validity of results in the eyes of editors of scientific journals. Unfortunately, the blind technique is impractical and wasteful; sometimes even counterproductive. It is wasteful and impractical because it forces me to look at large numbers of normal tissues with extraordinary care because I do not know that they came from a perfectly normal control.

How many times do we have to repeat that there is no such thing as a "normal" histological tissue structure? In every toxicity test I have evaluated, the normal pattern of the control group included a number of variations which could easily have been induced by the drug. Take the liver for an example. In some experiments the controls exhibit a distinct variation in nuclear size of the hepatocytes, in others high mitotic activity or large numbers of binucleated cells are present. In one study the protoplasm of the liver cells is dark and homogeneous, in another it is light, granulated, or vacuolated. Stainable fat may be present in single cells, not at all, or all over the place. Kupffer cells may be swollen, Glisson's triangles thickened, and inflammatory infiltrates, small granulomas or single cell necroses may be present in some liver lobes or in all. If these changes occur in the control group and if I know which slides are from untreated animals, I can save much time and concentrate on things which are not present in the normal controls.

I worry less about investigator's bias than I worry about investigator's indifference. Not to be told how the animal under review has been treated may reduce bias, but also dampens a professional's interest in the outcome of the study. I have never seen that lack of enthusiasm

and personal involvement of scientists has improved their performance. I also worry about a negative bias which crops up in every blind study and has its deep-rooted origin in the investigator's fear of making a false decision. Thus, any slight change which would be noted and registered if one knew that it was occurring in a treated animal may be suppressed subconsciously for fear of making an embarrassing error.

Moreover, blind technique and lack of investigator's awareness does not improve the accuracy of the pathological diagnosis. I work in a department of pathology which receives a large number of surgical biopsy specimens. If we had to look at them without knowledge of age, sex, primary disease and previous history of the patient, or if the clinicians would hide from us their diagnostic suspicions and speculations, the accuracy of our diagnoses would not be improved. On the contrary, we would probably miss many important findings.

One more thing must be added: There is no substitute for an experienced pathologist. You can submit your slides to an inexpert person, and he or she will make a mess of it, biased or unbiased. The good pathologist, however, will readily identify the abnormal findings. You facilitate his work if you indicate to him the slides which come from treated groups and those of the controls. He will also have no problem in recognizing those tissues where there is no difference between treated animals and controls. And then he will usually find some changes where he is not quite sure, where he feels that the drug has done something but where he is afraid that his personal bias and his keen scientific interest might lead him astray. For these tissues he will seek a second opinion or review the slides again, making sure that all precautions for blind evaluation will be observed.

With these arguments I find myself in agreement with the "philosophical considerations" Weinberger (1973) has submitted on the subject. The discussion will, of course, go on. All suggestions on how to improve the histopathological evaluation in toxicity testing are welcome, particularly when they come from experienced observers.

The histopathological evaluation of tissue slides involves a description of the morphological changes and, if possible, a semiquantitative rating of their severity. Because of the lack of a generally accepted nomenclature in toxicological pathology, we cannot be satisfied with simple diagnostic labels. All changes must be described in such detail that any pathologist can get a good mental picture. I even feel that unusual lesions should be documented, with photographs or, better yet, with a set of representative histopathological slides. The description of the lesion must, of course, not be repeated for each animal. Once it is defined, it receives an appropriate abbreviation. For each animal the pathologist notes whether or not the change is present and gives it a rating from 0 to 5.

0 : not present
1 : slight or questionable
2 : clearly present but not conspicuously so
3 : marked change
4 : severe change
5 : extreme change

Pathologists will in time develop their own rating scale which they should check occasionally by reviewing a set of slides more than once. There is no need to create a universally accepted rating scale. One pathologist may like the system given above, another colleague perhaps prefers a 0 to 10 + rating scale. We can still compare our results using the ridit method (Bross, 1958). With this procedure the empirical distribution of a treated group is used as the basis of comparison. By a simple calculation method which you can, according to the inventor, learn in 10 minutes, ridits are assigned to each degree of identified severity rating. In this way a word (e.g. "severe change") is replaced by a number (ridit) which is dependent on the position on the rating scale, the number of observations with a lower degree of severity, and the number of observations in the severity class itself. Thus if a different rating scale is used, the relative position of the individual observation vis à vis the whole sample should be the same.

To compare two groups of animals one calculates the average of their ridits, variance and confidence intervals. For a simplified method and additional statistical considerations, the original description of Bross (1958) must be consulted.

I must point out that more accurate morphometric methods do have a place in toxicological pathology, but in many cases the semiquantitative rating systems give perfectly adequate results. They should therefore always be tried first before you force a defenseless collaborator to spend much of his precious time measuring cell sizes and mapping necrotic areas. If you work for a very rich organization, you may have access to an automated image analyzer. This method of morphometric evaluation is easier and faster and very often proves to be a most useful procedure. But do look at the slides yourself anyway, you may see something the scanner was not programmed to find.

In presenting his findings, the toxicological pathologist should, as his major contribution, deliver a detailed description of the morphological changes. He should identify those which are surely or most probably due to the administered test compound. Any information on similar lesions induced by related or different agents will greatly facilitate the interpretation of the results. We must muster the courage to propose a reasonable explanation of the morphogenesis and try to identify the primary lesion which must be distinguished from the secondary reactions of the organism. We also should, as pointed out before, present evidence (not guesses) about the reversibility of all serious injuries.

The severity rating of morphological changes becomes most important when two or more related compounds are compared. The statistics used in such exercises depends on the nature of the data. Since pathological results rarely show a normal distribution, the t-test family is usually not appropriate. Chi-square test and distribution-free procedures, such as the Wilcoxon test, are therefore most popular with pathologists. In many cases the ridit method referred to above will prove to be useful. Its main advantage is the fact that comparisons are based on the empirical distribution of the data as they emerged from the actual experiment (Bross, 1958).

Apart from statistical significance biologists always look and hope for what one might call plainly evident obviousness. I am aware of the pain I have just inflicted with this pleonasm upon sensitive linguists. But their pain is minor compared to the one I endure when I see toxicological data, which are clearly, plainly, and obviously real,

being subjected to unnecessary statistical treatment. This pain is equaled by that inflicted upon me when I see how toxicological data, which are plainly and obviously of no biological significance, are twisted and torn with an array of statistical tests until one is found which delivers the magic 95% probability. One should accept the fact that certain experiments just do not produce useful results and that our experimental design was probably inadequate. Since good statistics rarely improve a bad experiment, it is better in such cases to admit defeat gracefully.

Toxicologists using the method of Discretionary Toxicity Study (ST I, 10) will often be rewarded with a set of data well suited for comparative evaluation. The approach involves sacrifice of groups of test animals at logarithmically-spaced time intervals, e.g., after 2, 4, 8, 16, etc., days. The drug-induced changes are then identified, described, and assessed semiquantitatively with an appropriate rating scale. After this is done, the fun part of the work begins - the search for the best method of presentation. First you may simply calculate the arithmetic means of the severity ratings for each lesion on each day of sacrifice. These figures are then plotted graphically as a function of time. Such a representation is instructive to demonstrate how rapidly-developing changes increase in severity on continued treatment with different doses.

It is sometimes possible, even preferable, to look at your complicated histopathological data as a quantal response, neglecting the severity rating of the responses. To do this, you merely note the presence or absence of the lesions. You then calculate the percent incidence for each dosage group at each day of sacrifice. The percent incidence is then plotted as a function of (the logarithm) of time. It is often possible to lump the results with all three dosage groups together and plot percent incidence against the cumulative dose (log dose x time). In doing this, one often finds an excellent correlation between incidence of a lesion and the logarithm of the cumulative dose. Litchfield (1949) has provided us with an excellent and simple method for the statistical evaluation of time-percent effect curves. It gives information on median effective time, the confidence limits and the slope function of the time-effect curves, all this (in the author's own words) within a few minutes.

The major advantage of this method, apart from allowing a quantification of histopathological results, is the simplicity of data acquisition. Instead of determining the severity or the extent of a lesion (which requires considerable experience), the observer only notes the presence or absence of the change. Once all types of lesions induced by the drug are identified by the pathologist, technicians may be trained to screen the slides simply for presence or absence of these changes, thus allowing a rapid preliminary evaluation of toxicity of a number of related compounds. Marked differences in toxicity between compounds can readily be identified by the percent incidence against (log) time or (log) cumulative dose.

The same approach may also be used to quantify toxic drug effects in man. Janku et al. (1971) have presented an elegant example: Psoriasis patients treated with various doses of triacetyl-6-azauridine were examined repeatedly for changes in hemoglobin concentration and white cell count. When the "effective times" for a drop in hemoglobin concentration and a leukopenic effect were plotted against the daily dose

on a bilogarithmic scale, a straight line relationship was obtained. Such data not only provide a rational basis for the determination of a safe dose, but would also be suitable for a rapid and quantitative comparison of related drugs, thus opening the way to a useful human toxicological bioassay.

DRUG COMBINATIONS - DRUG INTERACTIONS

Magnitude of the Problem

In Kühlewil, a small Bernese village situated at the foot of the Gurten, a little girl was wondering: "Mother", she asked, "are there people on the other side of the Gurten?" The mother answered: "Child, it is not up to us to reason." [1)]

As a fledgling toxicologist I received a similar answer from my superior. I asked him why doctors favored multiple drug therapy and fixed-ratio drug combinations. "Look", he said, "it is not up to us to reason. As toxicologists we just have to accept it and do our best to prevent disaster."

The prevalence of multiple drug therapy in clinical practice is amply documented in the published literature. There is no virtue in collecting another confirmatory survey. Many papers on this subject are written with an accusing or guilt-ridden undertone, reminding us that drug combinations are not anymore the hallmark of the skilful therapist. Worse yet, many of the most popular products offering fixed-ratio drug combinations are treated, at least by some influential colleagues, with outright contempt.

Nevertheless, many patients have more than one disease, each with an array of symptoms amenable to drug treatment. For them multiple drug therapy can be a blessing. As a witness I call on H.R. She is a 63 year-old lady who entered Oulu Hospital with an acute respiratory infection which was treated with penicillin and a cough mixture. As a diabetic and in cardiac failure, suffering from chronic pyelonephritis H.R. also received tolbutamide, digoxin, reserpine, furosemide, and potassium chloride. While in the hospital she needed a laxative and diazepam at night. Thanks to this combination therapy and good care the patient was able to return home after 12 days where she remained in good condition on 5 drugs (Sotaniemi and Palva, 1972).

1) The original version in Bernese is: "Lisi, mir wei nid grüble."

Drug combinations are also used at times to treat just one symptom. This is done in the hope of achieving superior therapeutic results. There is nothing wrong with this approach provided that it is done judiciously. Although I could cite many convincing examples, I must keep the balance. I have, therefore, selected a really bad one: it is about a 19 year-old obese but otherwise healthy man who was to be brought down to a tolerable weight in a hurry. He was given a digitalis-thyroid combination tablet, methamphetamine HCl to reduce appetite, a thiazide diuretic, and an insufficient amount of potassium gluconate. He developed severe muscle weakness, cardiac arrhythmia and died from ventricular fibrillation. It is not surprising that severe hypopotassemia combined with the increased cardiac irritability due to the thyroid hormone and the amphetamine greatly potentiated digitalis toxicity (Jelliffe et al., 1969).

It is clear from the two examples that drug combinations may be good or may be bad; the question to be asked here, however, is: are the bad ones frequent and serious enough to create an important toxicological problem?

If we survey an intensively treated hospital patient population and calculate the probability of potential adverse drug interactions, the risk appears to be staggering. Fortunately, not everyone who could develop a toxic reaction actually does so. Thus, the number of adverse effects developing due to the known and combined actions of more than one drug are not so frequent: the group managing the Boston Collaborative Drug Surveillance Program (1972) has intensively monitored 9.900 patients with 83.000 drug exposures. 3.600 adverse reactions were discovered of which 234 (6.9%) were related to drug interactions. This seems to be a rather small number which requires some explanation.

First of all, this figure of 6.9%, although it was arrived at by very careful investigations, must not be overrated. It includes, quite certainly, a number of toxic reactions which could have and probably were anticipated by the treating physician. On the other hand, it does not include as yet unknown drug interactions. The Boston group believes, - and recent findings tend to bear them out - that their number is probably quite large. We must suppose then, although conclusive statistics are not available, that adverse effects resulting from drug interactions constitute a significant toxicological problem which may yet present us with more upsetting surprises.

Pharmacokinetic Mechanisms of Drug Interactions

There is hardly a medical journal which has not, in recent years, featured a well written article on the subject of drug combinations. Most reviewers are fascinated by the concept of therapeutic blood levels, and many seem very disturbed by the discovery that this desirable state of pharmacokinetic harmony is often jeopardized if two or more drugs are administered concomitantly.

Not every pharmacokinetic muddle that is created by the presence of more than one drug in the body is translated into a significant adverse reaction. But if it is, it manifests itself either as an

exaggerated pharmacological and toxic action, or as an inhibition of therapeutic efficacy. An exaggerated effect may be expected if drug A enhances the absorption of drug B, if A displaces B from albumin binding sites (ST I, 34), competes for renal tubular secretion, enhances tubular reabsorption through a pH change in the urine (pages 45 - 49) or if A interferes with the metabolism of B. An inhibition of therapeutic efficacy occurs if A impairs the enteral absorption or the tubular reabsorption of B, if one agent stimulates the metabolism of the other (ST I, 28 - 33), and if A interferes with the transport of B to its cellular site of action.

Before leaving this subject, I must add one more comment: we sometimes forget that interactions in the pharmacokinetic phase of drug activity also occur if only one drug is given, provided that the compound is metabolized. Metabolites may vary in their pharmacokinetic properties just as much as drugs of different chemical structures do. Not only their half-lives in blood and tissues, but also their affinity to proteins, renal excretion, and effects on drug metabolizing enzymes are different from those of the parent compound. There is no reason, therefore, to look at drug combinations as an unique pharmacokinetic problem.

Interactions at Tissue Sites

This group of reactions to multiple pharmacotherapy includes those adverse effects which are not due to undesirably hight or low blood and tissue levels and not brought about by the pharmacokinetic interference of a second chemical. I am aware that a negative statement like this does not provide a satisfactory definition, but I want to leave it at that for the time being.

Included in this group of interactions at tissue sites are what the Boston Collaborative Surveillance Program (1972) lists as "cumulative pharmacologic effects," e.g. excessive central nervous system depression due to the administration of two or more CNS depressant drugs. Two hundred thirty of the 234 adverse drug interactions referred to before were of this type. This figure and my own impression from reading the literature convinced me that these interactions at tissue sites represent the clinicians' overt drug combination difficulty. The pharmacokinetic disturbances caused by drug combinations create fewer symptoms and could be looked at as our covert though not necessarily less important drug interaction problem.

Perhaps I should also say something about nomenclature of drug interactions. Toxicologists would use the pharmacological term "synergism" to describe the situation in which the toxic action of one compound is enhanced by another drug which by itself does not have this effect. "Addition" would stand for a combined effect of 2 drugs having the same mechanism and acting on the same receptor. "Supra-additive toxicity," still using the pharmacologist's terminology (Fingl and Woodbury, 1970), would be an enhanced toxic action exerted by 2 drugs which by themselves have the same overt effect but act by different biological mechanisms.

This terminology is by no means generally accepted and many other expressions such as potentiation, summation, and cumulative effects, are used frequently. This lack of uniform terminology is perhaps deplorable, but it does not constitute a major impediment for scientific communication. Where a biological mechanism of an adverse drug interaction has been elucidated a rigid classification is not essential. And for those interactions whose mechanisms are not known, even the most scientific classification system does not serve a useful purpose. Thus, we will keep our very simple subdivision which recognizes only two kinds of drug interaction problems: those which are explained by pharmacokinetic mechanisms and those which are not.

Animal Testing, Requirements

If you look through the published and the underground toxicological literature for guidance or perhaps even a ready-made experimental protocol, telling you how to go about preclinical testing of drug combinations, you will most probably be disappointed. But there are brief statements of two expert committees which, some ten years ago, elaborated an acceptable and sound concept.

The European Society for the Study of Drug Toxicity included this in their 1965 recommendations:

> "Mixtures of Drugs
>
> Before a new drug is made freely available, thought should be given to the other drugs which might be given concurrently with the new drug to the same patient. If it is likely that the new drug might often be prescribed along with another known drug, a check should be made that one does not exacerbate the toxicity of the other. It is clearly impossible to cater for all contingencies, but combinations which will probably be administered should be checked." (Davey, 1965).

This short paragraph still shows the unmistakable scars of a long bargaining session among strong-willed experts. But to the eternal credit of this committee the conclusion was reached that the problem should be approached first by giving thought to the potential adverse interactions of drug combinations. What they apparently recommended was a detailed review of the pharmacological and biochemical qualities of the drugs included in the combination, followed by some creative speculations as to how the substances might possibly interact. In a second phase, some of the more likely possibilities for interactions would then be "checked," i.e. tested in appropriate experimental systems.

In 1966 the WHO expert committee made, in effect, the same proposition:

> "Drug Interactions
>
> The effects of drug interaction should be studied when the new drug is formulated together with another drug, or when the initial trial in man requires the new drug to be given in addition

to concurrent therapy. Such studies may vary from a pharmacological appraisal to a complete toxicological evaluation."

The essential point of this statement is that the "pharmacological appraisal" of drug combinations must be a key step in the toxicological evaluation process. It is clear also that the WHO committee did not recommend a standard testing protocol but recognized that the approach must vary depending on the characteristics of the drugs involved and the projected circumstances of use.

That is the idealistic world of the experts; what now is the harsh reality facing the working toxicologist? The simple truth is that there _are_ standard procedures to follow and standard protocols to fulfill. The toxicologist is, of course, free to give as much thought as he likes to the potential interaction of his drug combinations, he may "check" it or "study" it in any way he can think of, as long as he has done the routine tests. He has also learned to accept the curious situation that two or more drugs put together in a fixed-ratio formulation are treated differently from a combination of the same drugs taken as individual products.

The fixed-ratio combination is, for regulatory purposes, treated essentially as a new drug and submitted to exhaustive toxicity testing. The ad hoc combination prescribed by practicing physicians is not subjected to any animal studies. Yes, the manufacturer is asked to consider potential interactions with drugs likely to be used concomitantly with a newly developed agent, but with the myriads of possible permutations only a small fraction can actually be tested in animals.

Let us first look at testing requirements for fixed-ratio drug combinations. In the now famous paper of Lehman written in 1959, these products are listed under "Category 2 - newness for drug use of a combination of two or more substances, none of which is a new drug." The paragraph summarizes what the US Food and Drug Administration considered adequate animal studies some 15 years ago. Since many regulatory agencies still adhere to this dictum and since few people actually had a chance to read the original version, it shall be reproduced here in its entirety:

> "Our standard requirement is a 90-day study in 2 species of animals, the rat and dog, employing the route of administration recommended for the combination. Numbers of animals are 20 rats and 4 dogs per group, in each of three treatment levels. If the background information is of sufficient completeness, perhaps the period of observation may be reduced to 30 days."

The paper also refers to drug combinations under "Category 3- newness of the proportion of a substance in a combination." In this statement which has apparently been forgotten, Lehman makes the reasonable suggestion that a new combination product using the same ingredients as a previous one but in other proportions need not necessarily be re-tested in animals. This is what the author writes:

> "It is quite likely that the data submitted in the original new drug application, if developed in accordance with recommendations for drugs in category 1 (newness for drug use of any substance, i.e. a complete toxicity study as outlined in ST I, 5 - 7), may be sufficient for evaluating safety of the new

combination. Label recommendations are important in deciding the extent of any additional work that may be required."

Also difficult to come by are the "Current Views on Safety Evaluation of Drugs" which Goldenthal published in 1968 in an FDA-sponsored publication. The requirements for combination drugs to permit phase I clinical trials are listed as: "LD_{50} by appropriate route compared to components run concurrently in 1 species." For phases II and III and New Drug Application a subacute toxicity study in 2 species of up to 3 months duration is required.

In the same publication Goldenthal makes some comments regarding "combinations of drugs" which I assume to be compounds used together but not included in the same pharmaceutical dosage form. The statement says:

"Combinations of drugs should be tested when there is evidence of toxicological or pharmacological interaction, even though each active ingredient has been tested independently and found to be without adverse effects."

The second part of the paragraph refers to fixed-ratio combinations:

"If one or more of the components in a combination product has shown an adverse effect on reproduction when tested individually, additional studies should be carried out on the combination."

That about sums-up the regulatory framework within which toxicological protocols for combination drugs and drug combinations have been developed.

Animal Testing, Procedures

The most commonly performed animal experiment for the evaluation of drug combinations is the LD_{50}. It is, as pointed out before, required for fixed-ratio combination products scheduled to be investigated in phase I clinical trials. It is also an easy way out for all those who are asked to give some toxicological information on the possibility that a new drug might cause an adverse reaction when given together with another agent.

The LD_{50} experiment must use the route of administration that is expected to be prescribed for man. One species, a small rodent, will be sufficient for most drugs. The performance of the test follows the routine procedure described earlier (ST I, 23 - 27). The single ingredients and the combination should be tested simultaneously.

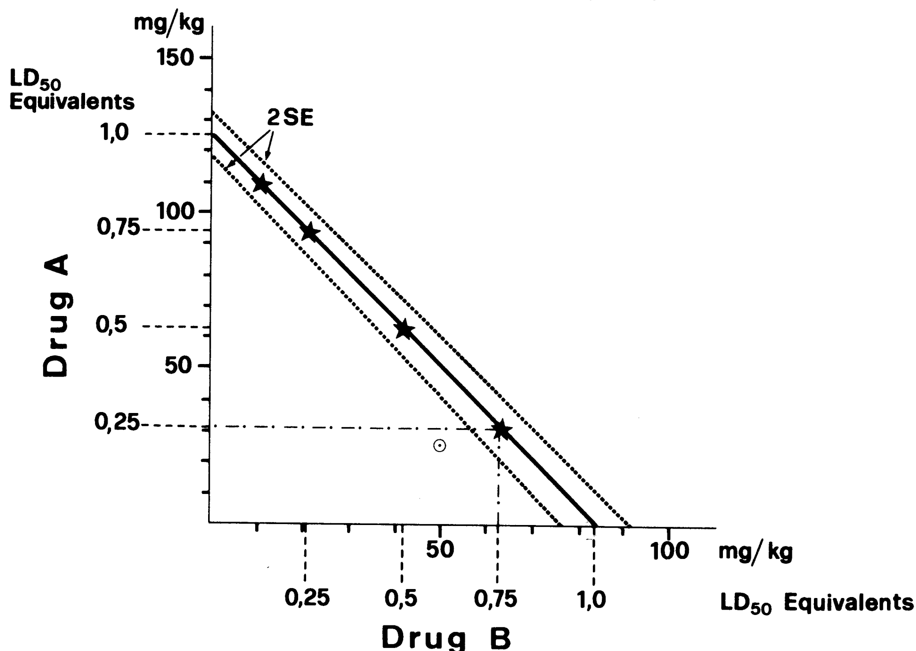
The only open question which needs some discussion is the determination of the dose ratios. For fixed-ratio combination products the conventional procedure is to attack the mice with a package which contains the goods in the same dosage ratio as that expected to be given to patients. This may sound logical but it is, and any pharmacokineticist will bear me out on that, scientifically inaccurate. The absorption and elimination kinetics of drugs in the small rodent are different

from ours. Moreover, the pharmacological interactions may differ depending on the ratio of the blood or tissue levels. Since it is rarely if ever possible to know how these ratios in mouse and man compare, one has no other choice than to test the combination at more than one dosage ratio. This appears reasonable also when we are concerned about possible adverse interactions of a single drug with other compounds likely to be prescribed concomitantly.

If one does LD_{50} determinations at various dosage ratios surprising differences may emerge. As an example an illicit but apparently popular combination product should be mentioned: heroin and cocaine. Addition of cocaine at low proportion antagonized i.v. injected heroin; at high proportions it potentiated heroin toxicity (Pickett, 1970).

This need for multiple dose-ratio testing makes our LD_{50} determination of drug combinations a formidable project costing perhaps as much as a medium-size automobile. For the selection of dose ratios the procedure of Chen and Ensor (1953, 1954) is recommended. It was developed for combinations with 2 components and starts out with an LD_{50} determination of the single ingredients. The figures are then plotted on the x-and y-axis of a coordinate system as shown below.

As a next step the two LD_{50}s are subdivided into LD_{50} equivalents (the LD_{50} of each component having the value of 1). If the toxicities of the 2 components are strictly additive, the LD_{50} of any combination should, when plotted on the coordinate system, sit exactly on the line drawn from the LD_{50} of drug A to the LD_{50} of B. As shown in the graph Chen and Ensor (1953, 1954) also plotted 2 standard errors of the LD_{50}s of drugs A and B and connected them with straight lines. This gives us an impression of the variability we may expect, but I would not like to speculate about the mathematical significance of these limits.



For our toxicological purposes it is sufficient to know that 2 drugs induce more than additive acute toxicity if the combined LD_{50} is clearly below the line linking the LD_{50} of A with that of B. (Point O on the graph). They antagonize each other if their combined LD_{50} lies above this line.

The coordinate system can also be used to select the dose ratios of drug combinations to be tested. The line linking the LD_{50}s of drugs A and B is then subdivided by selecting 3 or 4 equally spaced points (marked as x in the graph). The dosage ratios for these combinations is determined by drawing perpendicular lines to the x- and y-axis where the doses of each component can be read directly.

There is just one minor problem to be mentioned: The FDA protocol demands that the LD_{50} determination of the combination be run concurrently with that of the components (Goldenthal, 1968). This cannot be followed to the letter if we use the Chen and Ensor procedure. I would assume, however, that we comply, at least in spirit, with the FDA protocol, if the whole experiment is done within a period of several months, keeping in mind, of course, that experimental conditions must be kept unchanged.

The 30 to 90 day subacute toxicity experiment demanded by most regulatory agencies for fixed-ratio combination products does not involve unusual technical problems. It must be conducted with 3 dosage groups just as the tests done with single drugs (ST I, 5 - 8). The route of administration is the same as that proposed for man. Rats and dogs can most often be used, unless there are compelling reasons why another species should be preferred.

The selection of the dosage ratio is again the most difficult decision. One will probably not be questioned if one chooses to go with the ratio intended for the marketed product. But those knowledgeable in pharmacokinetics will not condone such a practice. They point out that steady state concentrations in body fluids and tissues are directly proportional to the biological half-lives of the drugs (Wagner et al., 1965, Levy, 1974). There exist, as everybody knows, very marked differences between biological half lives of drugs in animals and man. It is clear, therefore, that testing of a drug combination in an animal will often lead to ratios of blood and tissue levels which are vastly different from those obtained in man given the same mixture of active substances.

The logical conclusion would be to adjust the dose ratio for each species according to the pharmacokinetics of the components. This, of course, is extremely difficult and requires much information on pharmacokinetics in animals and in man. In addition, in each species we must expect various degrees of induction of drug metabolizing enzymes and competition for excretory mechanisms, and these factors are also influenced by the dosage.

The only practicable solution of the problem can be achieved by permitting adjustment of dosage ratios during the subacute toxicity experiment. One must know the ratio of the drugs present in the blood when steady state levels are reached in people treated with the combination. The animal experiment then aims at the same ratio, a goal which may at least be approached by blood level monitoring and dose adjustment.

The catch of the whole matter is that according to the guidelines, I must first perform a subacute toxicity test of the combination in 2 animal species before I am permitted to start multiple application of the product in man. Therefore, I do not have the information on steady state blood levels of the combination in man which would enable me to conduct a meaningful toxicity experiment. The routine subacute toxicity experiment performed with drug combinations containing the ingredients in ratio to be used in man must therefore be considered an unsatisfactory compromise.

Before suggesting alternate testing procedures, I must discuss a few more aspects of the subacute toxicity experiment. Should the test of a combination include groups of animals treated with the single ingredients? In the rare cases of new combinations for which toxicity studies have not been done with the single agents the compounds must be tested alone as well as together. In most instances, however, known drugs with a well-documented pharmacology and toxicology background are selected for combinations. If none of the active ingredients is known to cause any specific toxic effects, testing of the combination only is a reasonable approach. But if a compound is included which has a well-recognized organ toxicity the situation is different. In this case we want to know whether or not the addition of another drug enhances or reduces this specific toxicity. This, I am afraid, can only be determined if the experiment compares the effects of the single agents with those of the combination.

Relevance of Toxicological Testing

The guidelines on preclinical toxicity evaluation of drug combinations have now been around for some 15 years. During this time many combination drugs were studied and put on the market. An enormous pile of toxicological data must, therefore, have accumulated in the backrooms of industry laboratories and basements of regulatory agencies. To my knowledge nobody has made an effort to find out what these experiments have told us and how relevant they were.

Does it really make sense, any sense at all, to do LD_{50} determinations with drug combinations? What does one usually find, additive effects, potentiation or antagonism? What happened when the LD_{50} of a combination was lower than expected: which decision was made by the manufacturer and which by the regulatory agencies?

Have drug combinations which proved to be hazardous in man revealed anything in the batch of mice used to determine the LD_{50}? I know of one example, the combination of Na bromide and chlorpromazine, which produced severe coma when given to a hyperactive patient. Subsequent animal studies confirmed the supra-additive effect of the 2 CNS depressants. In acute toxicity experiments in mice, however, the LD_{50} of chlorpromazine was not altered by the bromide. (Norden and Plaa, 1963). Thus, at least in this case, the LD_{50} determination was of no use in predicting a dangerous drug interaction.

It is perhaps time that those who investigate drug combinations by the LD_{50} method consider whether or not they get their money's worth

for all that work. And what applies to the LD_{50} experiment is certainly true for the subacute toxicity tests.

In writing chapters like this on Formal Toxicology, I try to give a description of current guidelines and the actual practice of toxicity testing as it has developed during the last 10 to 20 years. Like many colleagues who have dealt with the subject, I keep repeating that guidelines are not protocols, that each drug product should be treated individually. Still, toxicological protocols - mine too - are usually written in a most conservative way, along the lines dictated by the most stringent guidelines. This practice, they say, will keep you out of trouble with regulatory agencies.

In general there is nothing wrong with doing one's work according to the books. In the case of drug combinations, however, the guidelines for toxicity testing are out of date. They are inadequate because they do not take into account the progress made in recent years in the understanding of drug interactions. For example, the pharmacokinetic mechanisms which explain a substantial proportion of all drug interaction problems in man are not, or only occasionally (and then indirectly), tested with the usual toxicity protocol. The interactions at tissue sites are recognized in certain cases with the help of the subacute toxicity study. But this experiment, as it is conventionally conducted, is inflexible and does not take advantage of many experimental techniques.

In the following paragraphs revised guidelines for the toxicological evaluation of fixed-ratio combination drugs and drug combinations are given. They are by no means officially recognized. Consider them as a basis for future discussions.

Guidelines for Evaluation and Testing of Fixed-ratio Combination Drugs

1. Fixed-ratio combination drugs containing only approved compounds

   -The pharmacological and toxicological properties of the active ingredients of the combination product are listed. Theoretically possible interactions are identified.

   -Animal experiments are designed to provide information whether or not the pharmacological and toxicological profiles of the active ingredients are altered when the components are administered in combination. Additional experiments test the likelihood that the theoretically possible interactions, as identified previously, would result in an undesired effect at doses corresponding to those used in man.

   -Available clinical experience on pharmacological and toxic effects of the single ingredients is summarized.

   -Clinical studies are designed to determine whether or not the pharmacological and toxicological effects are significantly modified when the drugs are given in the fixed-ratio combination.

- Available clinical information on the concurrent use of the active ingredients (in the form of single drug products) is summarized.

- The pharmacokinetic behavior (absorption and elimination kinetics, distribution characteristics of single doses) of the active ingredients and the combination are investigated in appropriate animal experiments. Possible competition for albumin binding sites is investigated in vitro.

- Steady state blood levels on repeated administration of the active ingredients and the combination are measured in appropriate animal systems, using the route of administration anticipated to be prescribed in man.

- Bioavailability of the active ingredients is tested in man after single administration of the final, pharmaceutically formulated combination product.

- The relevance of significant pharmacokinetic interactions observed in animal experiments is checked in human studies.

- Steady state blood levels and the pattern of the major metabolites in blood and urine of all active ingredients are determined in man.

2. Fixed-ratio combinations containing new, not approved active ingredients

- Complete pharmacological and toxicological studies of the single active ingredients are conducted.

- Phase I and II clinical trials of the single ingredients are completed.

- Further evaluation and testing of the combination follows the procedure outlined for fixed-ratio combinations of approved drugs.

Guidelines for Evaluation and Testing of Potential Drug Combinations Occurring During Clinical Use of a New, Single Entity Drug

- The drugs most likely to be used concurrently with the new compound are listed, together with their major pharmacological and toxicological characteristics.

- Theoretically possible interactions with the new drug are identified.

- The important pharmacological and toxicological properties of the new drug are identified. Animal experiments are designed to assess these effects in combination with the compounds most likely to be used concurrently with the new drug.

- In the list of compounds most likely to be used concurrently with the new drug those agents are identified which are known to cause

frequent toxicological problems in man, alone and in combination with other drugs. The effect of the new drug on the toxic actions of these notorious trouble-makers is investigated in appropriate animal experiments.

- In phase II and III clinical trials the therapeutic and toxic effects of the new drug are compared in patients with and without concomitant therapy. When an adverse interference is suspected, based on theoretical speculations, animal experiments or clinical observations, appropriate clinical pharmacological studies are initiated.

- Pharmacokinetic studies of the new drug in combination with another compound are considered for chemical reasons (e.g. chelating properties, strong protein binding characteristics, pH dependent excretion etc.) or to clarify unexplained changes of the pharmacological and toxicological profile induced by the concurrent use of a second drug.

- If the new drug is administered together with a compound for which a sufficient blood level is critical (eg. antibiotics) the pharmacokinetic work-up must include blood level monitoring of the second drug also.

Examples

The procedures outlined above attempt to concentrate toxicological testing of drug combinations on those areas where adverse reactions are most likely to occur. In order to identify these areas the total information package available on each active ingredient is surveyed by experienced scientists. Thus, an intellectual act is interposed between the decision to use 2 or more drugs together and the initiation of animal experiments designed to assess the safety of the new combination. With this proposal the recommendations of European Society for the Study of Drug Toxicity (Davey, 1965) and the WHO Expert Committee (1966) become mandatory requirements for the toxicological evaluation of drug combinations.

The type and the extent of the studies to be performed depends on the rational appraisal of the combination by the experts. It may turn out that the most appropriate experiment to characterize the toxic potential of the combination is a combined LD_{50}. I doubt that one would reach this conclusion often; but be it as it may, if it appears to be the best experiment, it should be done. Much more frequently one would be concerned about an enhancement of a pharmacological effect, e.g. excessive CNS depression, hypotension, bronchoconstriction, convulsions, prolongation of clotting time etc. If this is the case, animal experiments would be requested which would attempt to measure an alteration of these specific effects. Sometimes a drug's organotoxic qualities might be suspect and one would therefore develop an investigation which concentrates on functional, biochemical and structural changes of the suspected organ. Then again, one might be primarily concerned with pharmacokinetic interactions and concentrate one's efforts on blood levels and metabolites. At other times the scientific experts may

conclude that a given drug combination can safely be administered to man, and that the probability of an adverse interaction is remote. In these cases no animal experiments would be recommended.

I can just see the sceptical smile of the toxicology pros who do not dare to believe that one could get away with such a recommendation. I have recently seen a case which convinced me otherwise. It concerned a multidrug fixed-ratio combination product whose potential teratogenicity was to be evaluated. Unfortunately, I am not at liberty to reveal the details of the product. Suffice it to say that the sponsor of the drug assembled a well-documented survey on the experimental teratology data of all single ingredients and made reference to historical information regarding human use of the drugs. He then pointed out that the fixed-ratio dosage form made the administration of a meaningful dose of some of the active ingredients impractical, since it was expected that the pharmacological effect of other components would produce maternal toxicity and obscure the test results. He concluded that no useful purpose would be served by conducting teratology experiments with the combination product. An enlightened Anglo-Saxon drug regulatory agency concurred and accepted the application without animal experiments.

If animal testing of a drug combination is deemed necessary, the methods best suited to demonstrate the relevant effects should be used, and all members of the drug development team are mobilized for this purpose. If one of the components is known to be a weak convulsant the neuropharmacologist should see how the seizure threshold is influenced by the active ingredients and the combination. It is left up to him to propose the most appropriate animal system. If it is the function of heart and circulation we worry about, it is the cardiovascular pharmacologist's turn to come up with a relevant test model. An example is the study by Helfant et al., (1967) which demonstrated the combined depressant effect of procaineamide and acetyl strophanthidin on atrioventricular conduction in dogs.

The experimental pathologist will take care of suspected organotoxic effects. In such investigations we treat susceptible animals repeatedly with reasonable doses of single components and the combination, and use organ function tests, biochemical methods, and morphological studies to quantify the damage. A good example for such an experiment is the model developed by Linton et al. (1972) to evaluate enhancement of nephrotoxicity of cephalosporin antibiotics by furosemide. This problem, by the way, is also of some importance in man (Dodds and Foord, 1970). The authors combined various cephalosporins with a standard dose of furosemide using rats whose kidney function was stressed by administration of glycerol. Kidney damage was assessed from changes of blood chemistry and semiquantitative rating of renal tubular necrosis. Another example is the demonstration of severe hyperglycemia produced in rabbits treated with moderate doses of prednisolone and L-asparaginase (Khan et al., 1970).

The renal pharmacologist is consulted if we expect an undesired drug interaction leading to changes of salt and water balance. The experiments necessary to demonstrate the inhibiting effect of aspirin on diuresis produced by spironolactone (Tweedale and Ogilvie, 1973) are a good demonstration of how such a difficult problem may be solved with an imaginative experimental approach.

Before concluding the discussion of this subject, there are 2 remarks I want to make. First, the imaginative pharmacological and

toxicological approaches advocated for the study of drug combinations have, of course, been used for many years. Not much was published, however, perhaps because many of the tests gave the expected negative results. For example, Yelnosky et al. (1973) wondered whether their new diuretic metolazone would interfere with the anticoagulant action of heparin, and whether heparin would impair the drug's diuretic and chloruretic effect. They did a short term study in dogs, found no untoward interaction and put their worries to rest. Webb and Brandshaw (1971) were concerned by a clinician's suspicion that gallamine given to diazepam treated patients might cause prolonged muscle weakness and respiratory depression. They performed the appropriate experiment measuring the maximal twitch of 2 leg muscles after electrical stimulation of the peripheral sciatic nerve in cats, and concluded that diazepam did not interfere with gallamine and tubocurarine at the neuromuscular junction.

The second comment is a caution not to overestimate the relevance of animal models. One cannot just take a good looking experimental system from the literature, test 2 drugs alone and in combination and reach a valid conclusion on the presence or absence of an adverse drug interaction. There are good and relevant animal models, there are bad ones, and there are many more somewhere in between. Thus, as in all other areas of toxicology, the animal model is only a tool to help us resolve the problem of establishing safety of drug combinations. If it is used judiciously, it is a powerful tool, but only if it is properly integrated into the total drug development procedure.

About Human Testing of Drug Combinations

In this chapter the need for human testing of drug combinations was often mentioned. It is necessary when pharmacological and toxicogical experiments in animals give equivocal results. A certain toxic effect is seen perhaps only in one animal species and not in 2 - 3 others, or has been discovered merely at grossly exaggerated doses. In such cases careful testing in man is the best way to judge the relevance of the animal experiment. Even more important are human studies for all pharmacokinetic investigations which play such a vital role in the evaluation of drug combinations.

The problem is that we are rapidly running out of human resources for drug testing.

There is no question that better drugs for the treatment of important diseases are one of the major needs in medicine today. Most patients still available for drug testing must therefore be reserved for the evaluation of chemicals which are potentially useful for the treatment of as yet incurable illnesses. This leaves only a small number of research subjects for all the less urgent tasks. We must concede that not everything which one could and would like to check in man can be investigated in well designed clinical pharmacological studies. This is particularly true for the evaluation of potential pharmacokinetic and pharmacodynamic interactions of a newly developed drug with agents which patients may ingest concurrently. The number of such interactions is so large that only a very small fraction can be properly

assessed in man. A strict priority list must therefore be established. Theoretical considerations and animal tests will be most useful in helping select worthwile clinical study projects. Human experiments suggested merely to check a vague suspicion or to satisfy the whim of an overzealous individual must not be done.

If we cannot test every drug combination in man, chances are that we will miss a few truly dangerous interactions. The only way to recognize such hazards before much harm is done is careful and extensive monitoring of patients exposed to multiple pharmacotherapy. This is a very difficult and costly effort which only few medical centers are able and willing to undertake. The importance of this service is perhaps best demonstrated by the splendid record of performance of the Boston Collaborative Drug Surveillance Program. Of their many discoveries I only mention the recent demonstration that allopurinol combined with cyclophosphamide causes an excessively high incidence of bone marrow depression (1974). Another success obtained through routine checking of a hospital patient population was the finding of severe kidney lesions and renal failure in individuals treated with tetracycline and subjected to methoxyflurane anesthesia (Kuzucu, 1970, Procter and Barton, 1971). Another interesting observation, again made in regularly treated patients, was the abolishment of the hypotensive action of guanethidine, debrisoquine,and bethanidine by tricyclic antidepressants and several sympathomimetic amines. (Meyer et al., 1970, Misage and MacDonald, 1970, Starr and Petrie, 1972, Briant et al., 1973).

These examples show that, difficult as it may be, relevant clinical observations may be obtained in a mixed population of routinely treated patients. We must be aware that there are unexpected drug interactions which we fail to predict from animal experiments and from our knowledge of biological mechanisms. We must rely, therefore, on the astute observations of the practicing physicians who should certainly continue to use drug combinations but should always be on the lookout for as yet unknown adverse interactions.

III

SPECULATIVE TOXICOLOGY

DRUG-INDUCED LIPIDOSIS

General Lines of Investigation

This is a difficult subject for all of us whose premedical training dates back more than 10 years. Many of the basic discoveries necessary for its understanding have been made only recently, and some of the technical terms going with these discoveries have not yet become the universal property of biomedical scientists. A brief introduction may thus be helpful.

The first efforts to investigate drug-induced lipidosis were triggered by a morphological observation: in animal experiments it was found that a small number of drugs caused a bizarre foam cell reaction of the lungs, an accumulation of overstuffed cell elements filling most alveoles and small bronchi. More detailed studies uncovered similar cells emerging also in other organs, chiefly in lymphatic tissues. The electron microscopic observation of these foam cells disclosed the presence of many cytoplasmatic inclusion bodies. Most of them consisted of densely packed lamellae looking like the scribble of a toad's eye in a surrealistic drawing. Electron microscopists did not hesitate to label them as packs of phospholipid membranes tucked away in lysosomes.

This verdict sent the toxicologists back to the pathology books to learn more about the role of lysosomes in phospholipid metabolism. For once they were not disappointed. Much has been learned in recent years about the mechanisms by which these cytoplasmatic organelles take up and digest various macromolecules. Disturbances of these processes were recognized as the cause of the inherited storage disease (Hers, 1965). These conditions are characterized by ultrastructural changes identical to those seen in drug-induced lipidosis.

To pursue the problem further, the investigation was switched to a second level concerned with the chemical analysis of the foam cells' content. Histochemistry was tried first, since it was quite easy to wash out the foam cells and to subject them to chemical reactions. It turned out that they contained a mixture of lipids, chiefly phospholipids, sphingomyelin, plasmalogens, fatty acids, and cholesterol (Karabelnik et al., 1974). Having learned this much, it was important to obtain exact quantitative data on the lipids contained in foam cells,

lungs, and other tissues. Remember that organs have a distinct distribution pattern of phospholipids. Any marked change in this pattern could give a clue to the biochemical mechanism of the drug-related aberration.

The third level of investigation was concerned with metabolic aspects of the problem. It centered on the synthetic activity of the lungs, an organ which must produce phospholipids - surfactant - from the first to the last day of life. How and where are these lipids synthesized, how are they released into the alveoles and later disposed-of? How could drugs interfere with phospholipid turnover and what was the role of the drug metabolizing systems of the lung?

Finally there were toxicological problems to deal with. The question why some drugs, such as chlorphentermine, did and closely related compounds, such as phentermine, did not disturb lipid metabolism was puzzling. That nothing was known about the mechanism of the toxic effect was downright embarrassing. Whether or not species differences were prevalent was anybody's guess. And without these essential pieces of information how could one evaluate the clinical relevance of the experimentally-induced changes? The fact that this chapter must appear under the heading "Speculative Toxicology" reveals that not all answers have come forth, but enough data are on hand to appraise the problem and to develop some preliminary theories.

Morphology - Foam Cells and Myeloid Bodies

I call a foam cell any bloated, sick-looking cell element with finely granulated or vacuolated, pale-staining protoplasm in which some chemical substance has obviously accumulated. Its cytological pedigree often cannot be readily deduced by light microscopy. In other cases foam cells have retained an intimate contact with their surroundings and show morphological features permitting easy recognition as an original subunit of a given tissue.

When animals are treated with certain drugs, the first change which is noted within a few days is hyperplasia of granular type II pneumocytes with foamy transformation of the protoplasm (Heath et al., 1973, Karabelnik et al., 1974). Soon thereafter, masses of unattached foam cells appear. They are tightly packed in the alveolar lumen. One is amazed that the animals still are able to breathe. That these cells are lipid-stuffed macrophages has been settled definitively (Lüllmann-Rauch et al., 1972, Vijeyaratnam and Corrin, 1972). In later stages foam cells appear in lymph nodes, spleen, Peyer's patches, and other lymphatic tissues (Karabelnik et al., 1974). Their cytological nature is not yet clarified. Some are certainly macrophages, others may be reticulum and endothelial cells. These foam cells are often so numerous that they replace most of the lymphatic tissue. (Theiss et al., 1973).

Even more spectacular than the light microscopic changes is what appears in electron microscopic pictures. The alveolar macrophages are completely filled with myeloid bodies, single membrane-limited cytoplasmatic structures containing smooth membranes with lamellar or reticular arrangement. (Lüllmann-Rauch et al., 1972). In addition there are

other inclusion bodies with a very pretty regular, rectangular, or hexagonal crystalloid structure (Yates et al., 1967, Dietert and Scallen, 1969, Lüllmann et al., 1973, b). Inclusion bodies of the same structures, large numbers of them, appear in just about all other tissues. This comes as a surprise to the pathologist who would not have suspected such widespread changes from the light microscopic scrutiny of the organs. Inclusion bodies, by the way, are also present in leukocytes of the blood. This facilitates diagnosis of drug-induced lipidosis in man (Shikata et al., 1972). In many instances the cells containing myeloid bodies also show a hyperplasia of the smooth endoplasmatic reticulum (Hruban et al., 1972, Thys et al., 1973).

Myeloid bodies may also be induced in vitro. With chloroquine, for example, this was readily possible using mouse peritoneal macrophages. After incubation with 30 µg/ml for 15 min. to several hours, the autophagic processes were markedly activated and myelin bodies appeared (Fedorko et al., 1968). Similar observations were made with cultured dorsal root ganglion cells incubated with chlorpromazine (Brosnan et al., 1970). These observations indicate that an in vitro screening for lipidosis-including properties might be possible. Such simple tests are the dream of all toxicologists, but unfortunately, shortcuts are rarely possible. Pharmacokinetic and metabolic processes do not function properly in the in vitro system, and the animal experiment will still have to be done to confirm positive as well as negative screening test results.

Which Lipids are Stored?

Clean separation of lipids in biological material is a difficult matter. Each organ needs painstaking readjustment of the separation procedures. From those who have worked on this problem, we hear that the bulk of the lipids stored in foam cells and other organs of drug-treated animals are identical to those normally present in the tissues. Only some small and faint spots on the thin layer chromatography plates represent unidentified lipids not demonstrable in untreated animals.

The distribution pattern of the lipids in the organs is not, as one might perhaps expect, uniform. On the contrary, the chemical analysis has shown marked differences between the type of lipidosis induced by various drugs. After treatment of rats with 4,4'-diethylaminoethoxyhexestrol 2 HCl, for example, Yamamoto et al. (1971) detected a strange increase in the amount of lysobisphosphatidic acid which reached levels of 0.2% of the wet weight in the spleen. Normally this substance is only present in traces. What makes this observation particularly interesting is the fact that lysobisphosphatidic acid is also increased in Nieman-Pick's disease. The same phospholipid was also increased in livers of rats treated with high doses of triparanol (Adachi et al., 1972).

In our laboratory the levels of 8 phospholipids were studied in the lungs of rats. The fact that the results agreed well with data recorded in the literature confirms what was stated above: that each organ has a typical distribution pattern of its lipids which is little affected by changes in the environment. After treatment with the

anorectic agent chlorphentermine, an experimental antithrombotic drug RMI 10.393, and the psychotropic agent 1-chloro-amitriptyline, the levels of many, but not all, phospholipids increased markedly. Table 3 shows the distribution pattern and also lists the phospholipid content of isolated alveolar foam cells. It is readily apparent that chlorphentermine and RMI 10.393 induced similar changes, whereas the tricyclic antidepressant, 1-chloro-amitriptyline, differed quite markedly. This pattern is even more evident in the distribution of the neutral lipids which were present in moderate amounts in foam cells of chlorphentermine- and RMI 10.393-treated rats but abundant in those of 1-chloro-amitriptyline-treated animals (Karabelnik and Zbinden, 1975, a).

TABLE 3

Phospholipid Distribution in Lungs of Normal Rats and Lungs and Isolated Foam Cells of Rats Treated with Chlorphentermine, RMI 10.393 and 1-Chloro-amitriptylin, µg Lipid Phosphorus per Gram Wet Tissue (Adapted from Karabelnik and Zbinden, 1975, b)

Lipids	Normal lung	Lungs Drug-treated Rats			Foam Cells Drug-treated Rats		
		Chlorphent.	RMI 10.393	1-Chloro-amitript.	Chlorphent.	RMI 10.393	1-Chloro-amitript.
Lecithine	250	629	1052	362	1.833	1.688	1.054
Phosphatidyl ethanolamine	119	115	168	119	129	94	90
Sphingomyelin	57	62	77	50	129	102	76
Phosphatidyl serine	44	27	29	34	44	44	30
Phosphatidyl inositol	24	44	54	29	80	72	58
Phosphatidyl glycerol	11	44	54	16	110	123	72
Cardiolipin	7	0	0	6	0	0	0
Phosphatidic acid	0	16	23	14	32	45	48

One other thing is apparent from these data: the phospholipid distribution in the foam cells and in the lungs (from which most of the foam cells had been washed out) is quite similar. What was produced was also secreted into the alveoles and taken up by the alveolar macrophages. Neutral lipids, however, were not or only slightly increased in the lungs of drug-treated animals. They were present at moderate levels in foam cells of rats on chlorphentermine and RMI 10.393. In contrast, foam cells of 1-chloro-amitriptyline-treated animals contained considerable amounts of these lipids, particularly cholesterol esters and fatty acids. Thus, the disturbance of lipid metabolism and lipid secretion was quite different in rats treated with the psychotropic drug as compared to animals on chlorphentermine and RMI 10.393 (Karabelnik and Zbinden, 1975, a, b).

The Lung as a Center of Metabolism

Apart from its main job, gas exchange, the lung engages in a variety of metabolic side-actions. It is an active clearing-house for the turnover of biological polypeptides, amines, adenine nucleotides, prostaglandins, and foreign chemicals and synthesizes phospholipids which it needs for its own function in the form of the surfactant (Heinemann, 1973). Most of the phospholipids are manufactured in the type II alveolar cells. A key step is the incorporation of palmitate into dipalmitylglycerophosphorylcholine. Palmitate may come from the blood, but may also come from de novo synthesis for which the lung can engage various mitochondrial and non-mitochondrial pathways (Schiller and Bensch, 1971). The synthesized phospholipids are released by merocrine secretion into the alveoles where they are taken up by macrophages and disposed of through the airways and the lymphatics (Naimark, 1973). Not much is known about the control mechanisms of lipid synthesis, but it is relevant to know that it may be stimulated by chemical means, e.g., by L-thyroxine (Redding et al., 1972) and corticosteroids (Naimark, 1973). The drugs under discussion in this chapter produce an increase in certain lipids, phospholipids in particular. One could therefore speculate that they too might stimulate phospholipid synthesis. This is not, at present, a popular theory, but it should at least be mentioned since it may be resurrected one day.

The theory which is en vogue right now postulates some defect in the metabolic disposition of the lipids (Hildebrand et al., 1973). It has gained much support from the observation that histopathology and ultrastructure of tissues of animals treated with the incriminated drugs (the identity of all the culprits will soon be divulged!) are very similar if not identical to those seen in human storage diseases. These diseases are now recognized as inherited metabolic errors of synthesis of a lysosomal protein, an enzyme, or a structural protein and thus classified as "inborn lysosomal diseases" (Hers, 1973). The study of these storage diseases is a nightmare for our medical students, but for us who do not have to remember their many names, they are a fascinating subject and a promising lead to the understanding of the generalized drug-induced lipidosis.

It is particularly interesting to note that in storage diseases the stored material is usually heterogenous, although only one enzyme is missing. That is explained by the fact that the enzymes are not specific for one of the monstruous substrates but only for one type of linkage in the molecules. It follows that more than one enzyme is needed for the breakdown of the complicated lipids, and when one enzyme is missing, several substrates remain undigested (Hers, 1973). And something else is important: when the lysosomal digestion is perverted (to use Lloyd's (1973) expression) by a chemical substance or an enzyme defect, undigested material accumulates in lysosomes and may disturb other enzymatic processes. This may lead to an accumulation of secondary products, often in much greater quantities than those stored because of the primary damage (Lloyd, 1973). These factors may also account for the presence of so many different lipids in foam cells and other tissues of the drug-treated animals.

How then can drugs pervert lysosomal metabolism? They could, first of all, inhibit lysosomal enzymes. That this is possible was demonstrated in very elegant experiments by Davies et al. (1969, 1971). They "filled" rat liver lysosomes with I^{121}-labelled denaturated albumin, i.e., they injected this material i.v. to have it taken up by heterophagy into the cells and their lysosomes. The lysosome-rich fraction of the liver was then isolated and watched to see how it digested the albumin in vitro. The rate of production of the digestion product, iodotyrosine, could be measured. Pretreatment of the animals with acid proteinase inhibitors (trypan blue, aurothiomalate, and suramin), which are taken up by lysosomes also, slowed down lysosomal digestion of the labelled albumin. One could imagine that there might be drugs which act similarly on lipases within the lysosomes. This would then lead to an accumulation of the lipid substrates.

An elegant speculation of how this might happen was offered by Homewood et al. (1972). They note that chloroquine, a drug known to induce lamellar inclusion bodies in many tissues, has a pKa_1 of 10.2 and a pKa_2 of 8.1. At physiological pH 18% is present in the monoprotonated form, the compound is lipid soluble and capable of passing the cell membrane. The drug accumulates in lysosomes (Allison and Young, 1964). If it is correct that the pH inside the lysosomes is acid (DeDuve and Wattiaux, 1966), the compound will be converted to the doubly protonated form which is insoluble in lipids and unable to escape the organelle. This protonation of chloroquine would provoke some changes of the pH, and this in turn would impair the action of the digestive enzymes which are apparently easily upset by changes in their environment.

Another speculation, also developed on the example of chloroquine, was offered by Abraham et al. (1968). These authors assume that the drug initially labilizes the lysosomal membrane whereby enzymes would be released and autophagy of damaged cell organelles initiated. On continued treatment, the lysosomal membrane would become impermeable for enzymes so that their content would no longer be digested.

TABLE 4

Drugs Causing Generalized Lipidosis (or at least Myeloid Body Formation in Various Tissues)

Name (generic, trade, chemical)	Pharmacological class	References
chlorcyclizine	antihistamine	1, 2
homochlorcyclizine	antihistamine	1, 2
norchlorcyclizine	antihistamine	2
chlorphentermine	anorectic	3 - 6
cloforex	anorectic	7
fenfluramine	anorectic	8, 9
triparanol	anticholesteremic	2, 5, 9, 10
AY 9944, trans - 1,4-bis-(2-chlorobenzylaminomethyl) cyclohexenone 2 HCl	anticholesteremic	11
SC 12937, 20,25-diazacholesterol	anticholesteremic	10
Trimanyl R, 4,4'-diethylaminoethoxy-hexestrol	coronary dilator	12, 13
RMI 10.393, α - [p-(fluoren-9-ylidenemethyl)phenyl] -2-piperidine ethanol	exp. antithrombotic	6
iprindole	psychotropic agent	14
1-chloro-amitriptyline	psychotropic agent	6, 15
AC 3579, 2-N-methyl-piperazinomethyl-1,3-diazafluoranthen-1-oxide	psychotropic agent	16, 17
chloroquine	antimalarial	2, 18 - 21
paraquat *)	herbicide	22
clindamycin	antibiotic	23
erythromycin	antibiotic	23

*) Not a drug, highly toxic herbicide

References

1) Hruban et al., 1970
2) Hruban et al., 1972
3) Franken et al., 1970
4) Lüllmann-Rauch et al., 1972
5) Lüllmann et al., 1973a
6) Karabelnik et al., 1974
7) Magnusson and Magnusson, 1972
8) Lüllmann et al., 1973b
9) Yates et al., 1967
10) Dietert and Scallen, 1969
11) Kikkawa and Suzuki, 1972
12) Yamamoto et al., 1971
13) Shikata et al., 1972
14) Vijeyaratnam and Corrin, 1972
15) Theiss et al., 1973
16) Thys et al., 1973
17) Hildebrand et al., 1973
18) Abraham et al., 1968
19) Gleiser et al., 1968
20) Abraham et Hendy , 1970a
21) Abraham et Hendy, 1970b
22) Fowler and Brooks, 1971
23) Gray et al., 1971

Toxicological Speculations

A substantial number of drugs are known to cause foam cell reaction and generalized lipid storage in many mammalian species (mouse, rat, hamster, rabbit, dog). With other compounds the foam cell reaction of the lungs is not as dramatic, but lipid storage and myeloid body formation is found in many tissues. A list of these substances, which in all likelihood will be incomplete when this summary appears in print, is given in Table 4 . Many chemical classes and pharmacological groups are represented. Clearly, the ability to induce the syndrome cannot be considered a rarity anymore. Some of the drugs have never been used in man, some were marketed but withdrawn for various reasons, and some are still in use. It will not be long until our clinical colleagues and regulatory agencies will ask us probing questions. Thus, we must develop some guidelines on how to evaluate the toxicological significance of the phenomenon. We also should have some idea what to do if a new and promising drug ready for clinical trial is found to cause the foam cell reaction and lipid storage. What do we tell our bosses if they want to drop the hopeful candidate like a hot potato?

What we have to tell them first is that the process we are dealing with is not an aberration, or a lesion, or a degeneration, or a disease. There are hundreds of papers and many beautiful and expensive books which describe it as an absolutely normal turnover of cell constituents, a rejuvenation, if you will, by which the cell gets rid of worn out mitochondria and other organelles and delivers them to processing plants, the lysosomes, for recycling or elimination. This is called autophagy, a somewhat macabre term, but a perfectly normal process. Some cells also like to digest macromolecules coming to them from the outside. This is heterophagy. After the foreign materials have been taken up by the cells, essentially the same mechanisms and enzymes are used for their digestion.

We can follow these processes by looking at tissue sections with the electronmicroscope. The pictures are particularly impressive when the regular turn-over is upset as, for example, in hereditary storage diseases. Here the digestion of certain substrates, e.g., glycogen or phospholipids, is impossible. The material accumulates in lysosomes until the whole cell is filled with inclusion bodies and eventually dies. Similar pictures are seen when too much of the substrate becomes available. Examples of this are membranous inclusion bodies seen after crushing or sectioning nerves (Holtzman and Novikoff, 1965, Holtzman et al., 1967) and liver injury induced in rats with vincristine sulfate (Anderson et al., 1967).

Exactly how and why certain drugs upset the digestion of lipids in lysosomes is not clear. Lüllmann et al. (1973, b) have stressed the fact that foam cell-inducing compounds are "amphiphilic," i.e., they possess strong hydrophilic and hydrophobic groups at a short distance. They believe that this property facilitates entry and storage of the substances in the cells and permits binding to phospholipids, thus making phospholipids unassailable by the digestive enzymes. One could also imagine that once the drugs are inside the lysosomes, they may affect the enzymatic processes by some other mechanisms such as the ones suggested above for chloroquine. But whatever their action, important is the presence of the drugs at a certain concentration below which no disturbance of the digestive processes in the lysosomes occurs.

Experimental studies with foam cell-inducing drugs have shown that they need a critical concentration or dose to produce the tissue changes and the lipid storage. For some of the drugs this critical dose is above, and for some it is below the level which causes general toxicity such as weight loss, atrophy of organs, and death. Not all organs are equally sensitive. This means that myeloid bodies appear first in some organs and not in others. Whether or not this is just a reflection of the drug's tissue distribution is not known.

That critical dosage is important is confirmed by clinical experience with chloroquine. This antimalarial drug causes lipid storage and myeloid bodies in many tissues resulting in retinopathy (Hobbs et al., 1959) and myopathy (Gleiser et al., 1968). Zvaifler's (1968) review of the clinical literature clearly indicates that human toxicity related to myeloid body formation does not occur if the daily dose in adults does not exceed 250 mg.

Something else was learned from the hereditary storage diseases: the storage capacity of the cells for undigested substances and membranes is enormous. It may take months and years until functional deficits develop and cell death occurs. This means that drug-induced storage is also likely to be tolerated by the cells for an appreciable length of time. Since the storage process is reversible, no functional abnormality may ever occur if the treatment is discontinued before the cell is damaged. That is only true, of course, for a drug which is willing and able to leave the cell within a reasonable time. A compound remaining there for a long time is much more likely to lead to serious damage. There is one such compound known, the coronary dilator 4,4'-diethylaminoethoxyhexestrol 2 HCl, an agent marketed in Japan under the trade name of Trimanyl[R]. Man does not oxidize this compound whose half-life is extremely long. Tissue accumulation in the liver of patients having been treated for more than 6 months with 75 to 100 mg per day was as high as 1.160 mmoles per 100 g tissue or up to 0.74% of the wet weight (Matsuzawa et al., 1972). In several cases not only hepatosplenomegaly but also a fine granular liver cirrhosis was found (Shikata et al., 1972). Rats, on the other hand, excreted a hydroxylated metabolite. They also showed some lipid storage in tissues, but since the drug was excreted faster, no hepatosplenomegaly and liver cirrhosis occurred (Matsuzawa et al., 1972, Adachi et al., 1972). For the same reason lipid storage in rats was almost completely reversible within 8 weeks of discontinuation of treatment, whereas in patients the tissues still contained high levels of lipids and masses of myeloid bodies 1 1/2 years after withdrawal of the compound (Adachi et al., 1972).

From the experimental work with foam cell-inducing drugs and the knowledge of congenital storage diseases, it is quite clear that a cell which is completely loaded with myeloid bodies will eventually die. However, little is known about the functional consequences of moderate storage. I believe, but cannot prove it, that a slight to moderate increase in the number of myeloid bodies is of no significance for the cell. But even if everybody would share this belief, we would still have to prove it experimentally. It is important, therefore, that specific functions be assessed in animals with and without lipid inclusion bodies in many of their cells. In addition, cell survival and turnover should be measured to see whether or not the presence of myeloid bodies affects their life span.

To evaluate the toxicological risk of a foam cell-producing compound, we must determine the critical dose and tissue concentration leading to the disturbance of the lipid metabolism. We must determine the drug's half-life in the affected organ and the reversibility of the changes. We must measure blood and, if possible, tissue concentrations reached when therapeutic doses are given to man. Differences in drug disposition such as those found for Trimanyl^{R} must be looked for. Moreover, an effort should be made to monitor peripheral leukocytes in animals and patients for the presence of myeloid inclusion bodies.

There should be no objection to a drug's therapeutic use if it does not accumulate in human tissues, if blood and tissue levels in man are lower than those found to produce generalized lipidosis in animals, if the drug-induced lipidosis in the animal experiment is readily reversible and if, to make this perfect, no myeloid bodies are induced in leukocytes of treated human subjects. Compounds which cannot fulfill all these conditions may still be given to man, but the duration of treatment should be limited to a few weeks. However, until more is known about this strange drug reaction, I consider the ability to produce generalized lipidosis and storage of myeloid bodies (even if it occurs only at high doses in the animal experiment) an undesirable feature and would prefer (if a satisfactory one were available) a drug that is not burdened with this metabolic drawback.

IV

COMPARATIVE AND PHARMACOKINETIC TOXICOLOGY

GENERAL REMARKS

Pharmacokinetics is a relatively recent addition to those scientific disciplines which are considered essential for the preclinical evaluation of new drugs. At this time most toxicologists are still torn between the old loyalties for the classical standardized testing procedures and a new approach to safety evaluation in which pharmacokinetic considerations play an important part. Should, I am often asked, a certain drug be tested as admixture to the feed or by gastric gavage? If intubation is the preferred route, should the animals be treated five times weekly or every day? Or should perhaps more than one dose be administered per day? A satisfactory answer to these and similar questions cannot be given without knowing the drug's pharmacokinetic behavior in the animal species used and the absorption kinetics, blood levels, and excretion of the substance in man. Only then could an experiment be designed in which the exposure of test animals to the new compound be reasonably representative for that occurring in human therapy.

In the early phases of a drug's development, these facts are not known. At this stage the toxicologist must endeavor to expose the animals to fairly stable drug levels maintained over most of the day. The pharmacokinetic properties most likely to prevent the achievement of this goal are (in decreasing order of frequency): poor enteral absorption, rapid drug excretion, and drug cumulation. Unless one wants to take the risk of conducting a meaningless toxicity test or one in which many animals will die prematurely, it pays to have a word with the man in charge of pharmacokinetics and let him have a first look at the substance.

The same three problems, poor absorption, too rapid excretion, or cumulation, will also crop up when the compound moves on to clinical trials; but in human patients the possibilities for trouble are multiplied by diseases, genetic differences, and extreme variations in conditions of the drug's use. In this situation the pharmacokinetic facts learned in the animal experiments will provide most useful background information. The value of such knowledge not only for a rational therapeutic application of the new drug but also for the avoidance of preventable toxicity, will be illustrated in the following two chapters.

URINARY pH

A Rarely Considered Undesired Drug Effect

Nothing infuriates my small son more than a negative response from a bubble gum machine. He subjects it to a careful scrutiny and, after a well placed kick, rechallenges it with a second nickel. If the effect is again negative, he takes further action until the reason for the failure of the mechanism is elucidated or at least until he has recovered his 2 nickels. Why then does a negative response to a drug not ellicit from us a similar anger? Physicians have come to accept that some patients are seemingly untouched by their medication. In drug evaluation papers such failures of response get far less attention than the side-effects. Yet, from the sick patient's point of view, unreliable efficacy must be considered a very undesirable feature. The therapist therefore has an obligation to determine why the expected action has not occurred. Such findings are as important as those of unpleasant side-effects and should find a place in package inserts and drug monographs.

Mechanisms of Therapeutic Failures

Of the many possible reasons of non-response to a pharmacological agent, I suspect insufficient drug concentration at the site of action to be the most important one. It may be due to innumerable causes among which the bad habit of patients not taking the medication as prescribed must always be suspected. Poor absorption due to gastrointestinal disorders, insufficient bioavailability or decomposition of the compound in the gut may also prevent the build-up of effective concentrations where it matters. Excessively fast metabolism due to stimulation of microsomal enzymes has been identified as the cause of therapeutic failure in some cases (see ST I, 30). Perhaps as important or even more so, is accelerated drug excretion. And that is where urinary pH often plays a decisive part.

Why pH Affects Drug Excretion in the Urine

Movement of many drugs to and from their site of action, according to the "Bethesda Doctrine," may be explained by passive diffusion through a lipid membrane. Penetration through the membrane is only possible in the unionized form. Since most drugs are weak bases or weak acids and thus find themselves simultaneously in the ionized and unionized forms, the rate of penetration is dependent on the degree of dissociation, and this in turn is dependent on the pH. The dissociation characteristics of chemical compounds are expressed as the pKa, the negative logarithm of the acidic dissociation constant. (Brodie, 1964).

TABLE 5

Selected pKa's

Bases		Acids	
Acetanilide	9.3	Phenol red	strong
Theophylline	0.7	Benzylpenicilline	2.8
Caffeine	1.2	Ethyl biscoumacetate	3.0
Antipyrine	1.4	Probenecid	3.4
Benzocaine	2.8	Acetyl salicylic acid	3.5
Isoniazid	3.5	Ascorbic acid	4.1
Dextrorphan	4.8	Phenylbutazone	4.4
Aminopyrine	5.0	Sulfisoxazole	5.0
Papaverine	5.9	Sulfadimethoxine	6.3
Heroin	7.6	Sulfadiazine	6.5
Nicotine	8.0	Sulfathiazole	7.2
Morphine	8.2	Phenobarbital	7.4
Codeine	8.2	Thiopentone	7.6
Quinine	8.4	Barbital	7.8
Cocaine	8.4	Hydrochlorothiazide	7.9 and 9.2
Dextrorphan	9.2		
Chlorpromazine	9.3	Phenol	9.9
Ephedrine	9.6	Sulfanilamide	10.1
Atropine	9.6		
Amphetamine	10.0		
Tolazoline	10.3		
Mecamylamine	11.2		
Procaine amide ethobromide	strong		
Edrophonium bromide	strong		

Note: Various literature sources give slightly different pKa's.

The pKa

Why is it that this physicochemical constant, which is so important for a drug's biological behavior, is ostracized in the medical literature and banned from drug manuals and package inserts? Is it just the fact that we have difficulties to comprehend the meaning of negative logarithms? Taking the more familiar pH, also a negative logarithm, as a starting point, we may understand the meaning of the pKa better: it is identical to the pH at which the number of ionized molecules equals that of the unionized ones. Mathematically this is expressed by the Henderson-Hasselbach equation:

$$\text{pKa (base)} = \text{pH} + \log \frac{\text{ionized base}}{\text{unionized base}}$$

$$\text{pKa (acid)} = \text{pH} + \log \frac{\text{unionized acid}}{\text{ionized acid}}$$

The pKa of a weak base is low, that of a strong base is high; conversely, the pKa of a strong acid is low and that of a weak acid is high (Brodie, 1964, Cammarata and Martin, 1970). A list of pKa's of selected drugs picked out of the literature is given in Table 5. pKa's of many organic acids are also found in the collection of Kortüm et al. (1961). A companion publication for organic bases is the book of Perrin (1965).

How the pH Affects Urinary Excretion of Drugs

In acidic urine an acidic drug is present mostly in the unionized form and is reabsorbed to a large extent; with a basic drug the ionized fraction predominates and is excreted. In alkaline urine the opposite is happening, the acidic drug is mostly ionized and excreted, the basic drug unionized and reabsorbed. Conditions for pH-dependent reabsorption are particularly favorable if the pKa of bases is between 7.5 and 10.5 and that of acids between 3 and 7.5 (Milne, 1965).

Even small changes of the pH may have dramatic effects on drug excretion. A vivid illustration comes from West Africa: Students on a balanced protein diet excreted 40 to 60% of a single dose of amphetamine (a base with a pKa of 10.0) within 16 hrs. Their urine pH averaged about 6.0. Laboratory aides of the same institution, apparently less spoiled with meat, had a urinary pH of around 7.5. They excreted only about 5% of the same amphetamine dose in 16 hrs. Their amphetamine excretion was brought up to that of the students by a protein breakfast, whereas the students excreted very little unchanged amphetamine after taking it with Na bicarbonate. In addition, the students taking Na bicarbonate and the amine became more aggressive (Beckett, 1970). (Nothing is said in the paper how the high protein breakfast affected the technicians). Experimental studies in man with

C^{14} amphetamine also proved that acidification of the urine markedly accelerated the clearance of the drug from the plasma (Beckett et al., 1969).

In discussing these reports with my collaborators, I depicted the hypothetical example of an athlete who dopes himself with amphetamine. Through his gymnastic effort, his urine becomes acidic and the amphetamine is cleared in a short time, without ever reaching appreciable blood levels. The man might be caught by a urine check and punished although he never had any of the desired pharmacological stimulation. I was told, however, that "one" takes amphetamine together with Na bicarbonate, which only proves that we can make use of pKa's and pH's without knowing much about negative logarithms.

Aspirin, an acidic drug with a pKa of 3.5 gave us significantly higher blood levels upon i.p. injection into rats whose urine pH was lowered from 7.2 to 5.63 by treatment with ammonium chloride. With large doses of ascorbic acid the urine pH could only be reduced to 6.35, a drop of less than 1 pH unit. But this was sufficient to increase the aspirin blood levels significantly. The same may happen in patients whose urine pH is lowered because of a fever.

For the therapy of drug intoxications appropriate chemical manipulation of the urinary pH can become a useful procedure to accelerate excretion of the substance. This is one more reason why physicians should know the following 3 facts of every drug:

1. whether it is an acid or a base
2. what its pKa is, and
3. whether the excretion of the drug and its major metabolites is pH-dependent

Point 1 can be derived from the chemical formula. Below are the important chemical groups to help make the determination.

Acidic groups: $-O-SO_2OH$, $O-P-OH >$ $COOH >$ $-SO_2NHR >$ enol, phenol $>$ -COHN-CO

Decreasing acidic strength

Basic groups: guanido $>$ aliphatic amines $>$ aromatic amines and pyridine

Decreasing basic strength

The ionic strength of the above mentioned chemical groups may be influenced by other parts of the molecule. Moreover, many drugs have basic as well as acidic groups. (Example: Penicillamine which has a carbonyl group with a pKa of 1.8, a α amino group with a pKa of 7.9 and a β thiol group with a pKa of 10.5). This makes it often very difficult to determine how the compound will behave at a given pH. It

is sometimes also very tricky or even impossible to measure the pKa of a chemical substance. These problems are listed in order to show that things are not always as simple as one would gather from the discussion of the standard examples, such as amphetamine and acetyl salicylic acid. Just to know the pKa's of the acidic and basic groups of a drug is not enough. But it is often necessary to obtain the information listed under number 3: does the substance show pH-dependent excretion at extreme urinary pH values? For this, animal experiments must be conducted and experience in human subjects gathered. Manufacturers should include such information in package inserts, and books on therapeutics should make it readily available.

pH-Dependent Excretion of Metabolites

In the discussion of a paper by Beckett (1970) M. Sandler drew attention to the fact that urinary excretion of certain endogenous substances may also be modified by the pH of the urine. Among them are tryptamine, dopamine, and 5-hydroxy-indoleacetic acid. Ambitious research-tryptamine is determined to assess monoamine oxidase activity, dopamine excretion helps in the diagnosis of pheochromocytoma, and 5-hydroxyindole acetic acid in that of carcinoid syndrome- may produce erroneous results solely because of a failure to monitor urinary pH.

Other Toxicological Implications of the Urinary pH

One more example of therapeutic failure due to the wrong pH of the urine is that of methenamine, an old urinary antiseptic. To be effective, it must release formalin in the bladder urine, but can do that only under acidic conditions. The drug is ineffective if the urine is alkaline.

Severe toxicity occurs with drugs or metabolites whose water solubility is strongly pH-dependent. At unfavorable pH they can precipitate, block urinary flow in kidney tubules, and form kidney and bladder stones. The most dangerous of such substances are those which are given at high doses. Sulfonamides have a long-standing dubious reputation in this respect since they are generally poorly soluble at a pH below 6. Most of them have a very steep solubility curve and are highly soluble at a pH above 7. No wonder that toxicity studies of sulfonamides in rats whose urine is slightly alkaline usually do not disclose crystal deposits in renal tubules. That this can happen, however, was proven by a candidate drug N'-(4,5-dimethylpyrazolyl)sulfanilamide. This substance was poorly water soluble, even at alkaline pH. When fed to rats, it produced massive crystal deposits mostly in the renal pelvis. (Zbinden, 1964).

PERCUTANEOUS DRUG PERMEATION

Anatomical and Physiological Preconditions

The skin of an adult has a total surface area of 18.500 cm^2. The distance from the pretty outside to the microcirculation measures only 150 to 200 μ. The outer part consists of a brittle layer of keratinized remnants of epidermal cells which has pores up to 1000 Å wide. The more solid epidermis has intercellular spaces of 100 Å. Substances with a molecular weight of less than 10,000 can penetrate into and through the skin without too much difficulty (Stüttgen, 1972). In addition, there are hair follicles and sweat glands which seem to provide convenient shortcuts into the cutis. Here, I must hasten to say, the experts still do not agree on the importance of these preformed entry channels for the absorption of foreign chemicals (Scheuplein, 1967).

On the surface of the skin Nature has provided us with a protective layer of grease which we assiduously remove with soaps, detergents, and alcoholic sprays. A substance wanting to penetrate the skin nevertheless faces many obstacles: after the stratum corneum, whose inner compact layer appears to be the most important barrier, it encounters the two-layered epidermis, then a subepidermal membrane, the matrix of the cutis, and the walls of blood and lymph vessels. On the way to the blood stream, adsorption to many structural proteins may further impede the migration of the molecules (Stüttgen, 1972).

Does this complex barrier system give us adequate protection in a hostile chemical environment? People seem to think so considering the carelessness with which they apply brightly colored, scented and medicated ointments, lotions, and liniments to their skin. Remember that the same people worry a great deal about parts per million of chemicals, dyes, sweeteners, and preservatives, etc., in their food! This one-sided confidence in the protective function of the integument, however, is hardly justified. Take the example of the dog which had a small area (15 x 20 cm) of its skin painted with hydrazine. The poison was found circulating in its blood after 30 seconds, which indicates an almost instantaneous absorption. (Smith and Clark, 1972). Or consider the two Sepik brothers who had their Grilli treated with an alcoholic solution containing 20.7% salicylic acid; both died of salicylate poisoning a few hours after the second treatment (Lindsey, 1968). On

the other hand there is the report of a study on prisoner volunteers whose entire bodies were covered with 47.4 g of a 0.5% neomycin sulfate ointment. No traces of the antibiotic were detected in serum and urine (Panzer and Epstein, 1970). Thus, protection was virtually complete against neomycin and highly insufficient against the two other substances. This indicates that the barrier system of the skin is a discriminative one; a true challenge for the student of pharmacokinetics.

Factors Affecting Penetration of Chemicals Through the Skin

The endeavor to reconcile the observed movements of chemical substances into, through, and out of the body with the basic laws of physics provides much fascination for those who have made pharmacokinetics their lifelong concern. Their careful work on dermal absorption has already clarified many aspects of this complex process so that toxicologists and clinicians should be able to deal with the problem at least in a qualitative way. The theoretical explanation and the mathematical treatment of the percutaneous penetration of foreign substances, as a recent reviewer (Tregear, 1966) points out, still need much refinement.

Whether or not a topical preparation will cause a toxicological problem depends above all on the dose applied to the skin, the fraction of the dose which reaches the blood, and the rate of absorption which partly determines the blood levels. Drug absorption through the skin occurs by passive diffusion, both around and through keratinized and living cells. It is governed by Fick's first law which says that the number of particles moving through a plain surface area of 1 cm^2 is proportional to the concentration difference and depends on the diffusion constant of the penetrant. The complexity of the anatomical structure and many inevitable extraneous influences, i.e., mode of administration, vehicle, type of dressing, losses through washing or scaling, etc., can markedly affect the movement of penetrants through the skin. We must therefore not only know the diffusion constant of the chemicals but also be aware of the circumstances under which the barrier function of the skin may be weakened or break down entirely.

Just about anything we do to make the skin look cleaner, smoother, or more attractive increases permeability. This includes the use of depilatories, of which many contain thioglycollic acid (Wahlberg, 1972), all kinds of surfactants in detergents and soaps, and keratolytic substances (Tregear, 1966). Particularly effective is epidermal hydration (Stüttgen, 1972) as demonstrated in human subjects with methylethylketone. This substance (they use it to make smokeless powder) has a high vapor pressure and can thus be measured in the expired air by gas-liquid-chromatography. Applied to normal skin, it could be detected in 3 minutes; after soaking the skin for 2 hours with wet cotton pads, the stuff went in so fast that only 30 seconds elapsed until it appeared in the expired air. At the same time the subjects felt a burning sensation at the site of application (Wurster and Munies, 1965). You may have conducted a similar experiment on yourself when you applied a generous dose of expensive eau-de-Cologne to refresh your body after a hot shower. You remember, too, that the prickly sensation was different at various sites of your anatomy: The alcoholic refresher felt like water in the hollow of the hand, but it burned more or less intensely as it penetrated

into the skin at other parts of your body. Feldmann and Maibach (1967) have repeated this experiment for us in a more scientific fashion. Using C^{14}-labelled hydrocortisone they were able to compose a permeability map of the body surface. They found extreme variations in percutaneous absorption from the sole of the foot where a small fraction was absorbed to the scrotum where absorption was maximal. Similar regional differences were found with parathion (Maibach et al., 1971). Some of the data of these very elaborate experiments are summarized in Table 6 .

Table 6

Regional Differences of Dermal Absorption in Man

Site of Application	Hydrocortisone (1)		Parathion (2)	
	% absorbed	compared with forearm (ratio)	% absorbed	compared with forearm (ratio)
ventral forearm	1.04	1	8.6	1
foot arch, plantar	0.17	0.14	-	-
ball of foot	-	-	13.5	1.6
palm of hand	0.78	0.83	11.8	1.3
back	1.26	1.7	-	-
abdomen	-	-	18.5	2.1
axilla	3.7	3.6	63	7.4
scalp	4.41	3.5	32	3.7
forehead	7.65	6.0	36	4.2
jaw angle	12.85	13	33	3.9
ear canal	-	-	46	5.4
scrotum	36.2	42	101	11.8

(1) from Feldmann and Maibach, 1967. C^{14} hydrocortisone in acetone on surface of 13 cm^2 for 24 h

(2) from Maibach et al., 1971. C^{14} parathion in acetone, 4 mg/cm^2 for 24 h

Many drug absorption studies are done on the skin of the ventral forearm since this is a part which everybody will expose readily. Note that absorption there is quite poor, and certainly is not representative for the rest of the skin. The scrotum, on the other hand, laps up chemicals like a sponge. This explains perhaps why chimney-sweeps, although they are black all over, develop occupational skin cancer preferentially at the scrotum.

More factors which increase percutaneous absorption: high temperature has some effect and so has vigorous blood circulation. Such conditions develop under occlusive dressing, after imprudent exposure to sunlight, or therapeutic use of a rubefacient such as nicotinic acid benzylester (Stüttgen, 1972). Even more important for the physician is the fact that all lesions of the skin, from a simple abrasion of the corneal layer to burns, ulcerations, and neoplastic diseases, greatly increase permeability. A convincing example is that of boric acid which is poorly absorbed through intact skin. After application to a rat's back skin which was man-handled with a wirebrush, total absorption rose 34 fold (Nielsen, 1970). In psoriasis patients as much as 20% of C^{14}-labeled 5-fluorouracil administered in the form of a 5% ointment could be recovered from the urine. Only traces (0.27 - 1.12%) were absorbed through normal skin. Even higher amounts were taken up in patients with leg ulcers (approximately 50%), and in one case of spinalioma of the arm, as much as 61% of the applied radioactivity was recovered in the urine (Erlanger et al., 1970). Since 5-fluorouracil can depress the bone marrow and damage the intestinal epithelium, the total dose used for the topical treatment of skin diseases must be watched.

To assess the effect of various skin lesions, Carroll et al. (1967) made use of an elegant model system: they lacerated or burned part of a rat's tail, dipped it in a drug solution, and measured the uptake into the blood at regular intervals thereafter. Both lesions caused a marked increase in absorption of hexachlorophene, sulfadiazine, and potassium benzylpenicillin. To add an impressive clinical observation, the example of neomycin sulfate shall be used again. Although it did not, as mentioned above, penetrate the normal skin of excessively treated volunteers, it was absorbed and caused total deafness in a 9-year-old girl who suffered from dermatomyositis and numerous skin ulcers (Herd et al., 1967).

The Age Factor

The younger the skin the softer it is. We hardly need an experiment to convince us of the truth of this statement. Tregear (1966) has done it anyway and found in rat fetuses of 15 days that triethylphosphate penetrated the skin at a rate of 120 µcm/min. At a fetal age of 20 days penetration was reduced by a factor of 6, and it approached zero at the age of 90 days. This observation is not without practical implications as it points to the very high permeability of immature skin. That this is also the case in humans, Nachman (1970) has shown convincingly. He applied (perhaps accidentally?) phenylephrine HCl eye drops to the abdominal skin of premature babies and observed a localized blanching. He followed-up this observation and showed that the phenylephrine concentration needed to ellicit the vasoconstrictor effect and the time required for the white spot to appear were clearly related to gestational age. Babies having a gestational age of more than 38 weeks did not respond.

In summarizing the factors which increase dermal absorption, the following questions may be asked: What is young, warm, congested, and almost constantly wet? What is cleaned with detergents and wrapped round with plastic? What is often lacerated, inflamed, and even

ulcerated? What is treated with drugs and covered with cotton? The answer is simple - a baby's bottom. And that is also the reason why small infants are often the victims of percutaneous intoxications. In addition, very young babies are usually kept in groups so that accidents often occur in small epidemics. One such outbreak of disease was known already in 1886 and has since been observed from time to time. It usually occurs after an acute diaper shortage in a nursery which requires the delivery of an emergency supply. These badly needed new diapers are quickly stamped with the name of the hospital and used without delay. All babies suddenly become apathetic, cyanotic, and anorectic, and the finding of methemoglobin in their blood leads to the diagnosis: aniline intoxication due to absorption of the fresh laundry ink. (Kagan et al., 1949). A more serious accident occurring under similar conditions happened in another hospital which introduced the treatment of diapers with pentachlorophenol during the last cycle of the washing process. Twenty babies became severly ill and 2 died of liver, kidney, and heart damage as a consequence of dermal absorption of the antibacterial substance. (Armstrong et al., 1969). The recent tragic case of mass intoxication of infants treated with a powder containing 5% hexachlorophene was discussed previously. (ST I, 58).

The pediatric literature contains many examples of unusual intoxications as a consequence of dermal absorption of drugs. The one I like best is the story of an outbreak of precocious puberty in small infants which puzzled physicians in Chile until the unheard of occurrence of this rare syndrome in a brother-sister pair led to the diagnosis: the 7 infants who developed hyperpigmentation, mammary enlargement, vaginal secretion and bleeding, and growth of pubic hair had all been treated for ammoniacal dermatitis with the same locally manufactured ointment. This product was contaminated with estrogens. (Beas et al., 1969). Less unusual are changes induced by dermal absorption of anti-inflammatory corticosteroids. Fanconi (1962) tells of a case where 1% hydrocortisone ointment was used generously for the treatment of eczema; the child stopped growing completely until, after about half a year, the developmental defect was recognized and the treatment discontinued. Feinblatt et al. (1966) relate the history of a premature baby with epidermolysis bullosa who was treated with 1/4% hydrocortisone ointment. After only 4 days the patient gained weight excessively and developed Cushingoid features. Hydrocortisone, as will be shown below, is not readily absorbed through normal adult skin (approximately 1%). In mongoloid infants, however, 5 day absorption from a 1% ointment gently massaged into the skin reached 9.4 to 44.2%, and in mongoloids with eczema it was even higher (36.9 - 50%). (Feinblatt et al., 1966).

Chemical Structure and Vehicle

I could wind up the chapter at this point leaving you with the impression that any topical chemical we prescribe for our patients is likely to be absorbed, as long as it was used on damaged or diseased skin. However, those interested in the potential toxicity of cosmetics or other substances on normal skin must know more about the laws which govern uncomplicated permeation.

Of the chemical properties which determine the percutaneous diffusion process, molecular weight is not of great importance. Only very large molecules, proteins, and colloids, have difficulties being absorbed because of their size (Tregear, 1966). More important is the oil-water partition coefficient (Bartek et al., 1972). It is determined by shaking the test compound with equal volumes of phosphate buffer and an organic solvent, such as ether or heptane, at room temperature for 30 minutes. The concentration in one solvent is measured and the partition coefficient is calculated as follows:

$$\text{Partition coefficient (P.C.)} = \frac{\text{concentration of drug in organic solvent}}{\text{concentration of drug in buffer}}$$

This is not really much work, and it is difficult to understand why one has such a hard time to extract that figure from a chemist's files.

Substances having the tendency to accumulate in the organic solvent, such as the antifungal haloprogin (P.C. 45.4), usually penetrate the skin readily, whereas those with a low partition coefficient (e.g. the mucolytic agent N-acetylcysteine, P.C. 0.0004) are poorly absorbed (Bartek et al., 1972). This correlation must not be applied rigidly since there are apparently water-filled spaces in layers of the skin which also permit the penetration of water soluble compounds. A special case is caffeine which is rapidly absorbed although its P.C. is only 0.005. This drug is very soluble in organic solvents and water and can thus make use of all opportunities for easy travel. (Bartek et al., 1972). It would certainly be useful if manufacturers would provide information on oil-water partition coefficients and percutaneous penetration of all drugs and chemicals they sell. This information must be somewhere in their files but only very little of it is accessible in the medical literature. Table 7 contains such data. It is fragmentary to say the least. The figures are those obtained under special experimental conditions and might be quite different if the substances were applied in another solvent, at higher or lower concentrations, or in greater or smaller quantities.

The role of the vehicle has been studied extensively. The art to formulate topical preparations represents an important chapter in the curriculum of a successful druggist. Not that a substance could not be absorbed if it were applied to the skin as dry powder, but it goes better if it is made up in a suitable base. In general, absorption is increased if the vehicle contains a constituent in which the drug is readily soluble (Ostrenga et al., 1971). Boric acid, for example, penetrated lacerated skin of rats much more rapidly from a 10% methylcellulose jelly than from an ointment containing mineral and animal fats (Nielsen, 1970). The same author who reviewed the problem of boric acid poisoning was unable to detect any adverse reactions occurring after use of olagineous ointments in man which confirms the limited absorption of boric acid from such vehicles. If, however, a drug is very readily soluble in the solvent, much more so than in the biological tissue, it will resist diffusion out of the milieux which it likes so much. In such cases permeation through the skin is low.

Table 7

Penetration of Substances Through Intact Skin
(in % of Applied Dose)

Drug or Chemical	In Vitro Human Skin	Man	Rabbit	Rat	Pig	References
dinitrochlorobenzene		53,4 (1)				a
caffeine		47.6 (1)	69.2 (2)	53.1 (2)	32.4 (2)	a, b
1-naphtol		45.6 (3)				c
benzoic acid		42.6 (1)				a
dimethylsulfoxide		25-40(1)			100 (5)	d
p-aminobenzoic acid		28.4 (1)				a
salicylic acid		22.8 (1)				a
acetylsalicylic acid		21.8 (1)				a
butter yellow		21.6				a
methylcholanthrene		16.8 (1)				a
diethyltoluamide		16.7 (1)				a
testosterone	15.8	13.2 (1)	69.6 (2)	47.4 (2)	29.4 (2)	b, e
nicotinamide		11.1 (1)				a
haloprogin		11.0 (1)	100 (2)	96 (2)	20 (2)	b
K thiocyanate		10.1 (1)				a
parathion		8.6 (1)				f
dieldrin		8.0				g
malathion		7.8 (1)				a
urea		6.8 (1)				a
phenol		4.4 (1)				a
colchicine		3.7 (1)				a
salicylic acid -2'-hydroxyethylester		3.6 (6)	5.5 (7)			h
cortisone		3.4 (1)	30.3 (2)	24.7 (2)	4.1 (2)	b
hexachlorophene		3.1 (1)				a
N-acetylcysteine		2.43(1)	1.98(2)	3.5 (2)	6.0 (2)	b
chloramphenicol		2.0 (1)				a
nitrobenzene		1.5 (1)				a
hydrocortisone		1.0 (1)				i
fluocinonide	1-2 (10)					j
thiourea		0.88(1)				a

triamcinolone	0.6 (8)- 0.3 (9)		k
nicotinic acid	0.34(1)		a
5-fluorouracil	0.27 - 1.12(11)		l
androstenedione	0.26(12)		m
hippuric acid	0.21(1)		a
boric acid		trace(13)	n
neomycin	0 (14)		o
sulfadiazine		0 (15)	p
benzylpenicilline, K salt		0 (15)	p

Remarks concerning experimental conditions

(1) ventral surface of forearm; drug dissolved in acetone, 5 days
(2) back skin; drug dissolved in acetone, 5 days
(3) interscapular skin; ointment, 8 h
(4) elbow skin; drug in 80% gel, 4 h
(5) shaved back, minipig; in 80% gel, 4 h
(6) 1 h exposure
(7) guinea pig, 1 h exposure
(8) back skin, 30 h
(9) back skin; slightly covered, 3 1/2 h
(10) from DMSO, 11 h
(11) 5% ointment, under dressing, 3 days
(12) leg skin; 24 h
(13) in ointment or jelly, 5 - 8 h
(14) whole body; ointment, 12 h
(15) rat tail; 3 h

References

a: Feldmann and Maibach, 1970
b: Bartek et al., 1972
c: Harkness et al., 1971
d: Wong et al., 1971
e: Menczel and Maibach, 1970
f: Maibach et al., 1971
g: Maibach, cit. by Starr and Clifford, 1971
h: Pütter, 1970
i: Feldmann and Maibach, 1967
j: Coldman et al., 1971
k: Malkinson and Kirschenbaum, 1963
l: Erlanger et al., 1970
m: Oertel and Menzel, 1970
n: Nielsen, 1970
o: Panzer and Epstein, 1970
p: Carrol et al., 1967

A word should be said here about the wonder solvent dimethylsulfoxide (DMSO). For a time it was claimed that it had the ability of carrying almost everything through every membrane. After many disappointments specialists now know that this is not true. DMSO itself penetrates unbroken skin easily (Table 7), but it does not "carry" anything with it. However, it is an excellent solvent for many chemicals (Wong et al., 1971), causes hydration of the skin, and decreases viscosity of hyaluronic acid (Sneader et al., 1971). All these factors markedly improve the conditions for percutaneous permeation.

Drug concentration in the vehicle has something to do with the rate of permeation, as mentioned earlier. But satisfactory correlations of penetration with concentration are usually only found within a narrow range. For hydrocortisone butyrate, made up in a O/W cream, a relationship between concentration and penetration existed only between 0.05 and 0.5%. Further increase in concentration had no effect. (Ponec and Polano, 1972).

Toxicity Testing of Topical Preparations

Most toxicological guidelines demand that topical preparations be tested in acute and chronic experiments on animals with intact and broken skin. This is a cumbersome procedure, as anybody knows who has tried to put ointment on the back of a biting rat. It would perhaps be more logical to test the pharmacokinetic behavior of topical preparations first in an in vitro system. Excellent results have been obtained when penetration was measured on cadaver skin fastened over a suitable receptacle which was filled with buffer solutions. Loveday (1961) has used with equally good results the skin from the back of the ear of a freshly killed pig. In such systems a drug, perhaps radioactively labelled, is applied to the epidermal side of the skin preparation, and the fluid under the skin is sampled at regular intervals (Newbold and Stoughton, 1972). Table 7 contains the example of testosterone which confirms, as many experts claim, that the in vitro system can be used as a satisfactory screening method.

Still, government agencies demand animal experiments, and thus, animal experiments must be done. It is important to know, however, that there are remarkable species differences: the rabbit's skin absorbs more and faster than all other conventional laboratory animals. After the rabbit, in decreasing order, come guinea pig, rat, pig, and man. (Table 7) (Bartek et al., 1972).

Rabbits are fairly cheap, quite dumb, and remarkably meek. That is why they are used for percutaneous toxicity studies. Pigs, on the other hand, are smart, noisy, and expensive; but as far as the pharmacokinetics of the skin is concerned, they resemble us very much. It seems reasonable, therefore, to look at the pig as the animal of choice for dermal absorption and toxicity experiments.

Studies on dermal permeation in man have, as Table 7 indicates, been performed with a number of substances. These experiments were usually done with radioactively-labeled compounds. Fortunately, safer analytical methods have become available in recent years so that

studies which require the exposure of human subjects to radioactive chemicals may soon become obsolete.

In a few cases percutaneous drug permeation could be assayed with a biochemical method, e.g., the inhibition of dihydrofolate reductase by methotrexate appearing in the blood (Newbold and Stoughton, 1972). And for the penetration of the important group of anti-inflammatory corticosteroids, a bioassay measuring vasoconstriction in human skin was developed (McKenzie and Stoughton, 1962, McKenzie, 1962). With this test remarkable differences in vasoconstrictor activity were detected: Taking the effect of hydrocortisone acetate as 1, hydrocortisone alcohol had an activity of 0.1, and the phosphate and the sodium succinate were again considerably (10x) weaker. Triamcinolone acetonide (100), and fluocinolone acetonide (100) had the highest activity. For other steroids the activity was in the range of hydrocortisone, e.g., 9-α-fluorohydrocortisone acetate (1), dexamethasone (1), and methylprednisone acetate (1). (McKenzie, 1962). It is clear that this test not only measures skin penetration but also pharmacodynamic activities of the steroids.

Drug Metabolism after Percutaneous Penetration

The reason why one is not satisfied with a pharmacokinetic determination of the permeation of drugs through skin, but wants to see chronic dermal toxicity studies done at all cost, is the suspicion that the body may metabolize the compounds in the skin and that metabolites may be different from those formed after oral intake. There is not much known about such differences; as a matter of fact, with drugs which diffuse rapidly, the pattern of urinary metabolites has usually been the same as that seen after administration by other routes. Slowly migrating substances, however, may be metabolized in the skin. One of the few published examples is androstenolone sulfate which is apparently hydrolyzed in the skin, absorbed as the free steroid, and excreted as the glucuronide conjugate (Oertel and Menzel, 1970). Progesterone and testosterone are also markedly metabolized during their diffusion through the skin (Mauvais-Jarvis and Bercovici, 1972). A considerable delay in excretion, without change in metabolism was observed in man after dermal application of parathion (Quinby et al., 1963). If it is true that absorption should be slow for the skin's enzymes to get to work on penetrating foreign chemicals, it would be more appropriate to conduct such studies on pigs rather than on the traditional rabbit with its highly permeable hide.

GLUTATHIONE

Physiological Role

Glutathione (G-SH) was isolated from 50 kg lots of yeast and 20 liters of ox blood, and recognized as a tripeptide

$$
\begin{array}{lllllllllllll}
 & & & & & & & & & & CH_2 - SH & & \\
 & & & & & & & & & & | & & \\
H_2N & - & CH & - & CH_2 & - & CH_2 & - & CO & - NH - & CH & & \\
 & & | & & & & & & & & | & & \\
 & & COOH & & & & & & & & CO - NH - CH_2 - COOH & &
\end{array}
$$

by Hopkins. Modest as biochemists were in 1929 he did not speculate about the biological role of this remarkable molecule beyond calling it a "general cell constituent." When "Biological Redox Systems" became fashionable, G-SH found its convenient label; but even today the experts are still "looking forward to rapid and significant progress in many directions, finding out what the roles of G-SH and G-SH analogs are." These are the words of E.M. Kosower at the conclusion of the most recent (the third) International Glutathione Symposium in 1973.

It is not that the biochemists have neglected the strange and ubiquitous tripeptide. We now know that our body makes it in a two-step synthesis linking first L-glutamate to L-cysteine with the help of γ-glutamylcysteine synthetase. Glycine is added under the influence of G-SH synthetase (Meister, 1974, a, b). In the cell G-SH is oxidized enzymatically, making use of a G-SH peroxidase. It reduces naturally occurring hydroperoxides (Mills, 1960) and can, therefore, be considered as a protective mechanism for SH-groups in proteins.

Oxidized glutathione (G-S-S-G) must be reduced promptly to G-SH to be ready for another oxidative attack on the cell. This occurs rapidly under the control of glutathione reductase which takes its necessary H^+ from NADPH. In the red cell where G-SH enjoys the most renowned position, NADPH is exclusively derived from glucose through the pentose phosphate pathway.

Degradation of G-SH proceeds with the help of γ-glutamyltranspeptidase which produces cysteine-glycine and adds another amino acid to the γ-glutamyl moiety. The cysteine-glycine is split into its two components. From the other product a γ-glutamylcyclotransferase makes 5-oxoproline from which glutamic acid is formed enzymatically. It can re-join cysteine and thereby complete the γ-glutamyl cycle. This cycle is believed to play a role in the transport of amino acids across membranes (Meister, 1974, a, b).

There are fairly substantial amounts of G-SH in mammalian and other cells. Rat liver, for example, contains about 170 mg per 100 g fresh tissue (Boyland and Chasseaud, 1970). It is also present in blood (rat: 39 mg/100 g), in insects, yeast (128 mg/100 g) and even in potatoes (11 mg/100 g) (Boyland and Chasseaud, 1969). This finding should qualify G-SH as a major cell constituent. One can, however, live with little or no G-SH as demonstrated by the few individuals who lack one or the other of the 2 synthetases. Their main trouble, a part from being persued by blood-hungry scientists, is non-spherocytic hemolytic anemia (Boivin et al., 1974).

A much larger segment of the population has an enzymatic defect in the pentose phosphate pathway, usually glucose-6-phosphate dehydrogenase (6-GPD) deficiency. The G-SH levels in their red cells are low, making them an easy target for oxidizing drugs (Kellermeyer et al., 1962). I have found nothing in the literature about their G-SH levels in the liver.

I have recently listened to a spirited lecture of Prof. Jacques Monod on evolution. "L'évolution microscopique," that is what happened in the past on the molecular level, must be regarded, according to Monod, as a consequence of purely fortuitous events. The species inhabiting the earth today and all their complicated anatomical and biochemical make-ups, did not have to be like they are, but developed purely by accident. One could thus look at G-SH as a result of several random mutations which loaded cells with an exorbitant amount of a redox system, much more than needed to take care of the occasional hydroperoxide streaming by. Other mutations resulted in the creation of several glutathione S-transferases for which only recently a naturally occurring substrate, estradiol 17β, has been recognized (Chasseaud, 1974). Conjugation of estrogen certainly is not such a major vital function that it would justify the presence of so much G-SH in the cells.

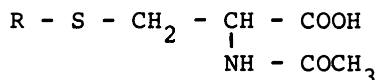
One may thus conclude that G-SH and its surrounding enzymes represent a powerful and costly installation, taking much space in the crowded cells but being of little immediate utility. They could be compared to the air-raid shelters which the Swiss build into every private home and which, a part from providing storage space for a few bottles of wine, do not serve a useful purpose.

When man came out of the woods and down from the mountain ranges, and began to produce and consume hundreds of toxic chemical substances, plenty of oxidizing agents suddenly invaded his body. The redox system which seemed to be an unnecessary luxury became an important protective device. Its significance was recognized by hematologists studying Favism and drug-induced hemolytic anemia (Kellermeyer et al., 1962). But its potential role for the protection against hepatotoxins also became apparent and has gained renewed widespread attention in recent years.

Protection against Hepatotoxins

G-SH effects its protective role in the liver with the help of a number of glutathione S-transferases. The process yields G-SH conjugates which are water soluble, anionic, with a molecular weight greater than 300. All these factors favor biliary excretion of the reaction products (Chasseaud, 1974).

Conjugation with G-SH also represents the first step in the synthesis of mercapturic acids which are detoxified excretion products of many foreign chemicals (Boyland and Chasseaud, 1969). Mercapturic acids are acetylated cysteine derivatives which are excreted in the urine and have the general formula



For more details about the fascinating story of the mercapturic acid biosynthesis, the review of Boyland and Chasseaud (1969) should be consulted.

The substrates for the glutathione S-transferases include a large number of electrophilic substances, an interesting collection of insecticides, herbicides, diagnostic reagents (sulfobromphtalein, BSP), carcinogens, and intermediates for many chemical production processes (Chasseaud, 1974).

The book which you are reading is supposed to report progress in toxicology. It is perhaps appropriate at this point to remind ourselves that progress was also made in earlier times. Mercapturic acids as excretion products of foreign chemicals were recognized almost 100 years ago by Baumann and Preusse (1879) and Jaffe (1879). Baumann and Preusse (1879) had one vigorous dog to which they could administer daily (with few interruptions) 3 - 4 g of bromobenzene for over half a year. In the urine they demonstrated the presence of bromphenyl mercapturic acid. Jaffe, also interested in the metabolism of benzene analogs, was told about the low toxicity of bromobenzene in dogs by Baumann who also gave him the permission to make use of this observation for his own work (so polite were the biochemists 100 years ago). Independently, Jaffe also isolated the mercapturic acid, which "fascinated him to a high degree."

The very important discovery that G-SH and not - as one could have assumed - free cysteine or cysteine incorporated in proteins, was the source that provided cysteine for mercapturic acid synthesis, was made almost 20 years ago (Barnes and James, 1957, Bray and Franklin, 1957).

The problem with toxicology today is that many important concepts are neglected because of the overwhelming interest of the scientific establishment in the microsomal enzyme system. And it is noteworthy that this widespread interest in the mixed function oxidase systems

has also been the major reason for the current attention paid to G-SH conjugation. The link was provided by the discovery of glutathione S-epoxidetransferase which takes care of the highly reactive intermediates generated from such compounds as polycyclic hydrocarbons by the microsomal mixed function oxidases (Boyland and Chasseaud, 1969).

No one will deny that modern man receives a generous portion of such chemicals through water, food, and the air he cannot avoid breathing. Thus, while I will not argue that the G-SH system happened during evolution purely through fortuitous mutations, I cannot but admire the foresight of fate which provided our early ancestors with a protective system that would help us, millions of years later, to survive the chemical revolution of the 20th century.

We now have enough facts to speculate about the role of G-SH as a protective mechanism against hepatotoxic substances. It is certainly clear that a low G-SH level in the liver is a most unfortunate condition for anybody unlucky enough to be exposed to hepatotoxins that are normally eliminated as G-SH conjugates or mercapturic acids. This includes the subjects lacking one of the two synthetases, and those with defective glutathione reductase. There are, however, only very few patients with these enzyme deficiencies and their reaction to hepatotoxic drugs and other chemicals is not known (Boivin et al., 1974, Löhr et al., 1974).

It must be mentioned that a malfunction of glutathione reductase is a frequently observed condition and is usually not due to an inherited enzyme defect. Glutathione reductase is a flavin enzyme with flavin adenine dinucleiotide (FAD) as prosthetic group. It is understandable therefore that impairment of glutathione reduction occurs as a complication of other diseases, particularly those which are associated with disturbance of riboflavin metabolism and intake (Löhr et al., 1974, Beutler, 1974). Supplementation with riboflavin will usually correct the biochemical abnormalities.

The relationship of riboflavin intake and G-SH levels underlines the role of nutritional factors and leads us to a very important aspect of the problem. Experimental data show that liver G-SH levels decrease dramatically during fasting. For example, rat liver G-SH dropped by about 40% after 1 day of food deprivation, and by 60% after 2 days. On re-feeding, normal levels were reached promptly, but when (and dairymen will not like this) a diet with 77% butter was offered and apparently also consumed by the rats, G-SH levels remained low (Maruyama et al., 1968). A marked decrease in liver G-SH was also found in rats kept on a low protein diet (Leaf and Neuberger, 1947) or on a diet containing only traces of cysteine and methionine (Lindan and Work, 1953). Two other dietary hazards causing a drop in liver G-SH are described in a French paper. One is concerned with G-SH in fois gras obtained from crammed geese. G-SH was between 80 and 92 mg/100 g of the precious raw material, compared to 186 to 238 mg/100 g of the liver of a normal goose. In the same laboratory some lucky rabbits received up to 4.85 l of wine in a period of up to 5 weeks. This resulted in a significant drop in liver G-SH. (Binet et al., 1937).

That a nutritional change in G-SH levels is of considerable toxicological significance was demonstrated by Jaeger et al. (1974): rats were exposed to the hepatotoxin 1,1-dichloroethylene, a compound used as a solvent and in the manufacture of certain plastics. This compound is activated in the liver and then conjugated with G-SH. At excessive doses it produces centrolobular liver necroses. And now the effect of starvation: the toxic level (LC_{50}), in normally fed rats of 15.000 ppm was down to 600 ppm after only 18 hours without food.

Systematic studies on the effect of poor nutrition on human liver G-SH have not been done. But if starvation has the same effect as demonstrated in the rat many of our patients may have dangerously low liver G-SH levels. And some of these patients may receive from us drugs that should be excreted as G-SH conjugates or mercapturic acids. It is important therefore, that we know the drugs which might be harmful to the liver under such conditions.

From these considerations we may conclude that our toxicological effort to detect potential hepatotoxins should also include a determination of G-SH levels in the liver. Barnes et al. (1959) have calculated that the turnover rate of G-SH in rat liver was about 42 mg/100 g of liver/hour enough, to maintain mercapturic acid synthesis at an appreciable level. The toxicological screening model designed to unmask potential hepatotoxic effects could then be made more sensitive by using animals with reduced liver G-SH. This can be done by depriving the animals of food or feeding a diet containing low protein levels or baker's yeast as the sole protein source (Lindan and Work, 1953). But for practical purposes it is more convenient to use certain α,β -unsaturated carbonyl compounds (Boyland and Chasseaud, 1970). Of these diethyl maleate has emerged as the toxicologists' preferred tool. In rats, for example, 0.6 ml/kg will lower liver G-SH to about 6% within 30 minutes. This by itself does not harm the liver in any way, but it renders it defenseless in the presence of toxins that should be inactivated by G-SH conjugation (Mitchell et al., 1973, a).

Low G-SH levels will also occur if one tampers with the G-S-S-G-reductase system. I have already mentioned as one possibility disturbances of the riboflavin metabolism. Another mechanism is insufficient supply of NADPH due to 6-GPD deficiency. This is probably only of importance in the red cell which derives all its NADPH from the pentose phosphate pathway. But it would be interesting to see whether or not liver G-SH levels of patients with 6-GPD deficiency are indeed as high as in normal subjects.

One can also damage glutathione reductase with chemical toxins. The standard compound to do this is ethionine. The exact biochemical mechanism of this reaction has not yet been elucidated. Ethionine is one of the classical hepatotoxins; one of the recent speculations proposes that high G-S-S-G levels shut off protein synthesis. If this is true, a link between the effect on glutathione reduction and liver damage from ethionine would be established. (Glaser and Mager, 1974).

We must now turn our attention to the substrate of the G-SH S-transferase system. The situation here seems quite straight-forward: there is only so much G-SH in the liver cells to be used for conjugation, and the synthetic enzymes can only generate a certain amount. If the hepatotoxins arrive in greater quantities, G-SH in the liver is depleted and vital SH-groups of proteins lose their protection. These facts were already demonstrated by Nakashima in 1934 who found a precipitous

decrease of liver G-SH levels in rabbits after an oral dose of 5 g naphthalene. Similar observations were made by Barnes and James (1957) who worked with 3,4-dichloronitrobenzene and related poisons.

From these observations it is readily understandable that liver poisons reacting with G-SH must show a clearcut dose-effect relationship. This however, is not the case for substrates needing enzymatic activation. Their hepatotoxic properties depend also on the ability of the mixed function oxidases to do their job. If one inhibits the microsomal enzymes, for example, with piperonyl butoxide, cobaltous chloride, aminotriazole or SKF 525A, even very large amounts of the hepatotoxic agents are tolerated without causing liver necrosis. On the other hand, stimulation of the drug-metabolizing enzymes potentiates the hepatotoxic qualities of such compounds (Mitchell and Jollow, 1974).

This interrelationship between microsomal enzymes and G-SH S-transferase system was demonstrated convincingly with acetaminophen, a drug which can cause liver necrosis in animals and man when given in excessive doses. The reason for this lesion is arylation of macromolecules by a reactive metabolite, believed to be N-hydroxyacetaminophen. This metabolite is conjugated with G-SH and excreted as acetaminophenmercapturic acid. Covalent binding of the toxic metabolite to liver cell proteins and liver necrosis occurred only after liver G-SH was exhausted. This process was accelerated in animals whose drug-metabolizing enzymes were stimulated by phenobarbital and inhibited when activation by the endoplasmatic system was blocked (Jollow et al., 1973, Mitchell et al., 1973, b,c, Jollow et al., 1974, a). The same happened with bromobenzene, another liver poison whose enzymatically activated metabolite is detoxified by G-SH conjugation (Jollow et al., 1974, b).

Finally one must also be aware of the possibility that the conjugating enzyme activity might be depressed in the presence of normal G-SH levels in the liver. This was observed in pregnant rats prior to delivery (Combes and Stakelum, 1962). This too, might be an additional risk factor should the animal be exposed to a hepatotoxic agent.

Clinical Relevance

When new toxicological concepts are enthusiastically accepted, when papers supporting them increase rapidly in number and sophistication, and when the disposable medical journals report them with big headlines, this is the time when practicing physicians are once again reminded of the rat-man gap, the enormous informational lag between easily obtained experimental data and comparable facts in man. It is one thing for an experimentalist to report that the liver G-SH in rats is lowest between 10 p.m. and 2 p.m. (Jaeger et al., 1974). But it is another thing to burden industrial physicians with the suggestion that night shift workers might therefore run a higher risk of suffering from hepatotoxic pollutants in the air. It was a simple study to knock off a few rats during the night time, all you had to do was to bribe a technician to stay in the laboratory over night and do the G-SH assays. It is practically impossible to gain comparable data in man. And that, my friend, is only one of thousands of animal experiments conducted year after year, one of many pieces of information with important potentional significance for man. It is a most difficult task in

toxicology to sift through the enormous experimental literature and pick out those reports that are of immediate importance for clinical medicine. In many instances we even lack the necessary knowledge to make a reasonable guess. It is not enough to do a toxicological experiment and dump it into the clinician's lap. We must provide him with facts if we think that our findings are relevant, and if we don't we should tell him that also.

This was a rather longwinded introduction to my attempt to gauge the clinical significance of the G-SH story. It was perhaps necessary since the rat-man gap here, too, is yawning.

What then can we say about the G-SH system in the human liver? One thing at least is clear: it appears to be operative. Its essentiality, however, is not undisputed.

The G-SH S-transferase system, although definitely demonstrated in man, is of low activity. If we rank the species in order of their transferase activities in hepatic tissue, we find ourselves placed between the pig and the chicken, clearly below all conventional laboratory animals and lower than the lizard but still above the duck, the goldfish, and the frog (Grover and Sims, 1964). In a recent study in which G-SH S-alkenetransferase was measured with 6 different substrates, Chasseaud (1974) found the activity of human liver to range between 2% and 33% of that determined in rats. On the other hand, G-SH S-alkenetransferase activity was present in appreciable amounts in human fetuses which were only 14 to 23 weeks old (Boyland and Chasseaud, 1968). But in rats transferase activity, measured as the BSP-glutathione conjugating enzyme, was very low before and at birth and reached adult levels only by the 7th postpartum week (Combes and Stakelum, 1962). These findings are important for pediatricians who may count on a functioning G-SH conjugation process already in neonates and very young babies. This was confirmed by Vest (1962) who found that the conjugated fraction of BSP in serum and bile of newborn infants was the same as in older children. Metabolic processes using the microsomal enzyme system, however, are insufficiently developed in young babies (ST I, 40).

Detoxification of drugs by cysteine conjugation and mercapturic acid synthesis was demonstrated in man. Subjects taking a dose of 2 g of acetophenetidine excreted it partly as cysteine conjugate and partly as the acetyleted product, the mercapturic acid. But these metabolites accounted only for 1.9 to 2.1% of the dose. In one subject receiving acetaminophen the conjugate accounted for 2.8% of the excretion products (Jagenburg and Toczko, 1964). Compare this with the 13 - 15% acetaminophen excretion as mercapturic acid demonstrated in mice and hamsters (Jollow et al., 1974a).

The rat-man gap becomes even more pronounced when we look at bromobenzene, a standard substance with which many of the experimental studies on the development of centrolobular liver necrosis have been performed. The compound was administered to 3 small children who received 1.5 g on 2 consecutive days (total dose 300 mg/kg). No mercapturic acid excretion was demonstrated in the urine, whereas this metabolite accounts for 20 to 40% of the dose in laboratory animals (Wainer and Lorincz, 1963).

These observations prove that important species differences are complicating the extrapolation of the animal experiments to man. But

even among laboratory animals there are differences in the activity of the various enzyme systems contributing to mercapturic acid synthesis. One frequently cited example is the very limited excretion of mercapturic acid in guinea pigs. In this case the evidence points to an insufficiency of the acetylating process, i.e. the final step in mercapturic acid formation. The initial conjugation with G-SH and the hydrolysis of the conjugate to the S-substituted cysteine, glycine, and glutamic acid seems to function well (Bray et al., 1959).

Species differences are also recognized in the area of the mixed function oxidases. This is important for those compounds which must first be converted to reactive metabolites before conjugation with G-SH takes place. The fact that rats are much less sensitive to acetaminophen than mice and hamsters, according to Jollow et al. (1974, a) is probably due to a slower generation of the reactive metabolite. These facts must be kept in mind when we speculate about the possibility that enzyme induction could accelerate the formation of reactive metabolites in the human liver. It is quite clear now that large amounts of drugs are needed to initiate such an induction. Not every compound which demonstrated its ability to induce enzymes when given in huge amounts to small rodents will have an influence on mixed function oxidases in man when given at the much more modest therapeutic doses. Thus, enzyme induction may play a role in man but only in those cases where inducer molecules have invaded the liver in sufficient quantities.

We also need large doses of the hepatotoxins to deplete liver G-SH. In experiments with acetaminophen it was shown that covalent binding of the active intermediate to liver proteins and liver damage occurred only when about 70% of the liver G-SH was depleted (Mitchell et al., 1973, a). To do this we need large amounts, much more than necessary for therapeutic purposes.

G-SH conjugation as a minor pathway in drug metabolism becomes important only when so much of the compound is ingested that the major pathways are greatly overtaxed, e.g. after a suicidal attempt. From such cases we have recently learned an impressive lesson that G-SH-related detoxification is indeed operative and quite important in man. Prescott et al (1974) who are apparently plagued by frequent misuses of acetaminophen, have treated 7 cases of severe intoxications not with G-SH (which penetrates cell membranes badly) but with cysteamine. And miraculously all recovered without liver necrosis, showing only occasional and slight liver function abnormalities. Eleven similar cases not treated with the sulfhydryl compound developed severe liver necrosis. Thus, it is quite clear that for acute intoxications G-SH conjugation and mercapturic acid synthesis represent an important detoxification mechanism not only for animals but also for man.

Much more difficult is the question regarding the clinical significance of slightly lowered G-SH levels in the liver, such as they might develop in the course of various liver diseases and poor nutrition. Do they represent a risk factor per se or in combination with other injuries to the liver such as a drinking bout, a halothane narcosis, a virus infection? Should we worry about drugs such as ethacrynic acid (Porter, 1966) which are chiefly excreted as cysteine conjugates and thus continously drain-off cysteine from our G-SH pool? Should we be concerned, to come back to an example I mentioned before, about the factory workers frequently exposed to small quantities of solvents and chemical intermediates that are eliminated as mercapturic acid? Should

the determination of mercapturic acid excretion or liver glutathione levels be part of our routine diagnostic procedure? An answer to these questions cannot be given. But the dramatic illustration of the importance of the liver G-SH system as provided by the illustrative animal experiments has certainly given the clinical investigator food for thought and an impetus for meaningful research.

V

PHARMACODYNAMIC TOXICOLOGY

HYPERGLYCEMIA

To Test and to Evaluate

Technological advances and automation have made rapid strides in the business of clinical pathology. Many laboratory tests are now done by ingenious machines which turn out results so fast that one needs other machines to store and retrieve the scientific riches. Experimental toxicology has created huge processing plants in which hundreds of urine, blood, and tissue samples are analyzed. Gone are the days when attractive and compassionate technicians insisted that you accompany them to the animal room to inspect an anemic rat or a jaundiced dog. Gone are the days when test animals had names and when somebody not only recorded their poor appetite and scruffy fur, but tried desperately to find the cause of the trouble. In these early days of toxicology, testing and evaluating went on simultaneously. Reports were written to provide either impassioned support or final condemnation for a test compound. Today, they are not much more than annotated computer print-outs delivered to somebody's desk to be analyzed and evaluated.

Evaluation of toxicity data today has become a serious matter. It involves, first of all, a value judgement of the experimental design and of the adequacy of the experiment's execution. To do this, something must be known about the people who conducted the test and the competence of those who supervised the work. It is erroneous to believe that toxicity testing is simple and straight-forward work which anybody can perform. Experiments done by incompetent investigators are often worthless, and so are those in which technical failures, poor health of the animals, uncontrolled conditions, and poor record keeping have introduced incalculable variations. It is thus necessary that the quality of toxicity data be rated. Should two studies give conflicting results, the better one should be given more weight for the final evaluation.

Evaluation of toxicity data must always consider all the facts known about the test compounds. It is wrong to zero-in on one single aspect of a drug's action without considering the total of its chemical and biological qualities. And, finally, evaluation of toxicity data cannot be done without considering the purpose for which the drug is proposed, and the patients likely to be treated.

In evaluating toxicity data, one is always handicapped by the paucity of responses with which the organism can manifest its discontent with the actions of a chemical substance. Biochemical parameters can change upwards and downwards, and the ability of tissues to respond to injuries with morphological changes is also quite limited. There is no list of acceptable toxic symptoms, and no one must say that a certain effect observed in animals automatically precludes the drug's administration to man. But there are signs and symptoms which upset toxicologists more than others and which we do not want to dismiss lightly. Hyperglycemia is one of these red flags. It is a symptom which is observed frequently, particularly in early acute and subacute toxicity studies. It can be a totally insignificant consequence of a stress effect, or it can be an ominous sign of chemical destruction of Langerhans' famous islets. With hyperglycemia toxicologists cannot be content, as they are with many other changes, to record the observation silently. It is not enough to bring it casually to the attention of the clinical pharmacologist. To see hyperglycemia means to initiate additional pharmacological studies, to determine the mechanism of action, and, if this is not possible, to define the circumstances under which blood sugar levels might also be changed in normal as well as diabetic human subjects.

Pharmacological Mechanisms

Carbohydrates in the proper composition and skillfully prepared make the most delicious meals of all. So that we may indulge with impunity in sweet sugars and crisp amylopectins, we employ two major hormones, insulin and glucagon, and keep most of our liver cells oscillating between storing of excess provisions and supplying glucose to hungry tissues. Brain centers monitor and regulate the complicated mechanisms. When we are in an aggressive mood, they dispatch generous amounts of epinephrine to bring our blood glucose levels up to full combat readiness. The kidney plays an important part also: it possesses an efficient reabsorption capability to prevent losses of precious fuel. In addition, it has a rather primitive and incompetent emergency overflow mechanism to protect the tissues against excessive glucose levels.

The most frequent form of hyperglycemia, apart from the postprandial variety, is probably that due to catecholamines, be they released endogenously or administered as therapeutic agents. The effect is brought about by an activation of liver and muscle phosphorylase and by an inhibition of insulin release from the pancreatic islets (Porte and Robertson, 1973). Excessive stress can therefore produce a metabolic state similar to diabetes with hyperglycemia and reduced glucose tolerance. This explains why stressful procedures such as acute and subacute toxicity tests in animals, and accidental

intoxications in man are often associated with hyperglycemia. Examples for epinephrine-mediated hyperglycemias are: the acute curare effect in rabbits (Szabó and Ferenczy, 1967), blood sugar rise following a sudden drop in blood pressure during i.v. infusion of nitroprusside in dogs (Staquet et al., 1965), prolonged elevation of blood glucose and glycosuria seen in man after acute poisoning with organophosphate insecticides (Namba et al., 1970), and probably also hyperglycemia encountered in rats during urethan anesthesia (Hantschmann, 1972), after solanine poisoning (Satoh, 1967) and oral administration of aminopyrine (Amano and Takaori, 1968), and that seen in rabbits after injection of pentamethylenetetrazol (DeBonnevaux et al., 1968).

The exact pathways by which catecholamines and stress induce hyperglycemia are not always readily apparent. Their basic effect is activation of phosphorylase which is mediated by cyclic AMP as second messenger. This is, at least in mice, initiated by stimulation of adrenergic α receptors (Arnold and McAuliff, 1971). The effect is, however, not inhibited by α receptor blockers, whereas some (not all) β blockers antagonize the hyperglycemia (Innes and Nickerson, 1970). The best inhibition of epinephrine-induced hyperglycemia in dogs was found when α and β blockers were used together (Nash and Smith, 1972). Thus, not only α but also β receptors seem to be involved. This is also apparent from the observation that blockers of β receptors inhibited hyperglycemia produced by injection of histamine (Classen and Späth, 1968). Stimulation of β receptors with substances such as isoproterenol and soterenol also caused hyperglycemia, together with an increase in lactic acid levels. The effects were blocked by β antagonists (Aiggilo et al., 1969).

The whole situation is complicated by remarkable species differences. This is illustrated by the following observations: The α-blocking agent phentolamine produced hypoglycemia in rats, dogs, and man as one would expect. In these three species norepinephrine had no metabolic effect. In fasting rabbits, however, both phentolamine and norepinephrine caused hyperglycemia of considerable magnitude. To complicate things a bit, phentolamine also inhibited norepinephrine-induced hyperglycemia (Calvey, 1969).

The situation is just as hazy with regard to the action on insulin release. Catecholamines inhibit insulin release via stimulation of pancreatic α receptors. At the same time they stimulate insulin release through an effect on a β receptor, this action being mediated via generation of cyclic AMP. (Porte and Robertson, 1973). Knowing this, it becomes understandable why the β stimulant isoproterenol was less effective than epinephrine as a hyperglycemic agent in intact rabbits, although it was more potent with regard to glucose release from liver slices in vitro. In alloxan diabetic rabbits where insulin release did not come into play, isoproterenol was also more potent than epinephrine as a hyperglycemic agent (Potter et al., 1972).

Dopamine and L-DOPA must both be included in the list of hyperglycemic substances, but L-DOPA acts only after being decarboxylated to dopamine. As far as the mechanism of action is concerned, it is inhibition of insulin release from the pancreas which is supported by the most convincing evidence (Håkanson et al., 1967).

Toxicologists who have difficulties explaining hyperglycemia induced by stress or by substances structurally related to catecholamines can take comfort in the conclusion of two reputable experts who called

the metabolic effects of epinephrine and its congeners "confusing" (Innes and Nickerson, 1970). As things stand now, we must often be satisfied with a partial answer. We can link the hyperglycemic effect of a drug to some interference with sympathetic function, with storage or release of catecholamines, or with generation and breakdown of cyclic AMP. But since these are fundamental systems and processes which are activated through many different mechanisms, it will often not be possible to demonstrate with certainty the sequence of events leading from the administration of a drug to the appearance of the hyperglycemic response.

This generalization will probably not satisfy a pure pharmacologist. But for a poor toxicologist who cannot spend the rest of his career unravelling a biological mechanism, it is of substantial help. The knowledge that a drug-induced hyperglycemia is clearly mediated through endogenous catecholamines, dependent on the presence of intact adrenals or normally functioning adrenergic receptors, will help him enormously in assessing the human risk and in restoring his own peace of mind. Two examples, a new and an old drug, will demonstrate how helpful the demonstration of catecholamine-connection can be.

Put yourself first in the position of the toxicologist who works on a very promising new hypotensive, Catapres^R (2-(,6-dichlorophenyl-amino)-2-imidazoline hydrochloride). The clinical pharmacologist is ready to start his trials in man when suddenly your inquisitive associate discovers a "diabetogenic effect" in rats, hyperglycemia, glycosuria, reduced glucose tolerance, low serum insulin level all included. I am not sure that the situation was actually as dramatic as that, but I am convinced that everybody concerned with this new agent was relieved when the effect on glucose metabolism was shown to be due to α adrenergic stimulation (Rehbinder and Deckers, 1968, Senft et al., 1968) and therefore not likely to cause any problem in the clinic.

The old drug example is morphine-induced hyperglycemia. This effect has been known since the pre-classical times of pharmacology. In this case hyperglycemia is due to catecholamine release from the adrenal gland. The example is interesting because morphine activates epinephrine release via a specific central receptor. When the drug was injected into the ventricles of a cat, it produced hyperglycemia with a fiftieth of the dose necessary to do the same job by i.v. route. Since this remarkable observation was made, the hunt for the exact location of the receptor is on. It was long suspected to sit in paraventricular areas (Borison et al., 1962), but results of a new search seem to locate it in the central gray, in the floor of the 4th ventricle, or even in structures near the ventricular surface of the brain stem (Feldberg and Shaligram, 1972).

Morphine is not the only substance which can claim an epinephrine-mediated, centrally induced hyperglycemia. The same effect has been demonstrated for 3-acetylpyridine (De La Mora et al., 1968) and 2-deoxyglucose (Frohman et al., 1973). The latter compound, however, interferes with glucose metabolism also in a much more fundamental way. As a non-metabolizable analog of D-glucose, it blocks competitively phosphoglucoisomerase (Wick et al., 1957). This leads to hyperglycemia and lowers intracellular glucose levels. From this example we must learn that a substance can produce the same symptom, hyperglycemia, by two completely different mechanisms.

A group of drugs which is widely used and frequently associated with disturbances of glucose metabolism is that of the benzothiadiazine diuretics. The major symptoms, hyperglycemia, glycosuria, and reduced glucose tolerance, are familiar to all practicing physicians. To claim that the exact mechanism of action of this metabolic aberration is known would be a misrepresentation of the facts, as anybody who follows the voluminous literature or who has read the comprehensive discussion published by Losert (1968) will attest. The speculations go in many directions, an interesting one being that of Losert (1968) who suggests that the diuretics inhibit 3',5'-AMP-phosphodiesterase, the enzyme which destroys cyclic AMP. Through this effect more of the second messenger would be available to activate phosphorylase and glycolysis. Much research is also conducted on the importance of low potassium levels as a cause of hyperglycemia. It is probable that disturbance of the electrolyte balance by the diuretics has something to do with the development of hyperglycemia since potassium supplementation alleviated the benzothiadiazine-induced elevation of blood glucose (Rapoport and Hurd, 1964). But the cause-effect relationship is not quite so simple; for example, a clinical experiment showed that significant hypokalemia induced in human volunteers by hydrochlorothiazide was not associated with impaired glucose tolerance (Wales et al., 1967).

In recent years two new diuretics have been introduced: ethacrynic acid and furosemide. They, too, elevated blood sugar in animals (Foy and Furman, 1967). In man hyperglycemia did not seem to occur quite as often as with the benzothiadiazines. However, the two newcomers are not totally innocent as both have been associated with a number of cases of glycosuria and impaired glucose tolerance (Wales et al., 1968 b, Cooperman and Rubin, 1973). Mechanisms suggested for the explanation of these effects include an influence on insulin-dependent enzymes in the liver, a reduction of glucose utilization through a direct effect on adipose tissue, and a decrease in insulin-like activity in serum perhaps mediated through catecholamine release (Lebacq et al., 1967, Cooperman and Rubin, 1973).

The champion hyperglycemic agent of the thiazide series is diazoxide. Its potent hyperglycemic effect, particularly in combination with a benzothiadiazine diuretic, became evident early in the clinical trials. At that time Dollery et al. (1962) pinpointed the most likely mechanism of action: inhibition of insulin secretion through a direct action on the islet cells of Langerhans. Although other possibilities have been discussed, the demonstration of low serum insulin levels following i.v. and oral glucose load is good evidence for the proposed pharmacological explanation (Seltzer and Allen, 1969). That α adrenergic stimulation with reduction of cyclic AMP in the pancreas could be responsible for the action on insulin release is possible (Senft et al., 1968).

As always, when an interesting new chemical appears, a good number of structural analogs of diazoxide were synthesized. Many lowered blood pressure and inhibited insulin release, but interestingly enough, not all seemed to reduce urinary output as much as the standard drug (Wales et al., 1968, c). Chlorthalidone, another diuretic which is chemically somewhat different from the benzothiadiazines, was also occasionally associated with hyperglycemia in human subjects (Topliss et al., 1964) but did not measurably inhibit insulin release (Wales et al., 1968, a). A close analog of this drug, however, 1-oxo-3-(4'-chlorophenyl)-3-hydroxyisoindoline (C3/76) resembled diazoxide in its pharmacological

actions including the effect on the β cells of the pancreatic islets (Wales et al., 1968, a).

This short survey has made it clear that hyperglycemia as a symptom is shared by a number of structurally or pharmacologically related chemicals. The best defined effect, inhibition of insulin release, is clearly evident with some of these agents but by no means with all. Other mechanisms suggested above come into play and may assume the most decisive role for the rest of the compounds. It must be pointed out, however, that inhibition of insulin release is not the privilege of diazoxide and related hypotensives and diuretics. A compound as different as the seven carbon sugar D-mannoheptulose has shown the same biochemical property (Coore et al., 1963), and so has the popular antiepileptic agent diphenylhydantoin.

Diphenylhydantoin is one of our older drugs whose therapeutic usefulness has not been matched by any of the newer antiepileptic agents. For the toxicologists, it has been a steady source of startling surprises; to the clinicians, one of the most versatile inducers of puzzling toxic reactions. Among these unexpected events we also find hyperglycemia. It was not unexpected that convulsions, induced chemically and by electric shock, would send up the blood sugar levels. In animal experiments diphenylhydantoin blocked the convulsions but not the hyperglycemia. As a matter of fact, it produced high blood glucose levels after i.p. injection in rabbits (Belton et al., 1965) and i.v. administration in dogs (Sanbar et al., 1967). In a recent series of experiments in mice using the ED_{50} of its anticonvulsant effect (5 mg/kg), diphenylhydantoin was shown to impair glucose tolerance and to inhibit the hypoglycemic effect of tolbutamide (Mennear and Gossel, 1973). Results of various studies in patients on high doses of the anticonvulsant correlated well with the animal work (Peters and Samaan, 1969). In a patient on 400 mg of the drug plasma insulin was low. After an i.v. injection of tolbutamide, no insulin was found in the plasma, whereas plenty was there when the experiment was repeated after withdrawal of the drug (Fariss and Lutcher, 1971). But to make the story more complicated, I must mention that diphenylhydantoin-induced hyperglycemia in mice was abolished by adrenalectomy (Mennear and Gossel, 1973). This shows that we cannot get away from the fact that blood sugar regulating systems are interdependent. Although biochemical pharmacologists have found out a lot about drug-induced hyperglycemia, there is still much opportunity for stimulating speculations (Editorial, 1973).

The next group of drugs which disturb glucose metabolism includes a number of hormones. The most familiar changes for the clinician are hyperglycemia and glycosuria occurring with glucocorticosteroids. It is generally agreed that these effects are due to stimulation of gluconeogenesis and hepatic glucose production, reduced glucose utilization in the periphery and some facilitation of renal glucose excretion (Boyer, 1967). In animals corticosteroid-induced hyperglycemia can best be demonstrated after partial pancreatectomy. In dogs with 84 - 87% and in rats with 95% of the pancreas removed, hyperglycemia developed in a few days after oral administration of dexamethasone and triamcinolone. Doses as low as 0.1 mg/kg in dogs and 0.25 mg in rats were sufficient (DeCorral Saleta et al., 1959). I found this report very interesting since it fits well with the clinical impression that glucocorticosteroids affect glucose metabolism chiefly in the presence of pancreatic deficiency.

Estrogens also induce a disturbance of the glucose metabolism. This was clearly demonstrated in the largest ever group of drug taking volunteers, women on oral contraceptives. The major symptom is abnormal glucose tolerance leading to high postprandial blood glucose levels and an excessive response in the corticoid-stimulated glucose tolerance test. The effect is dependent on duration of drug intake and probably occurs in more than half of all women taking the pill. The mechanism is not clearly understood although much points towards some change of glucose uptake and glycogen mobilization in the liver (Javier et al., 1968), perhaps through an influence on corticosteroid metabolism (Elgee, 1970). Conventional oral contraceptives consist of a mixture of an estrogen and a progestational agent. As far as side effects are concerned the estrogens have always been looked at as the bad guys. In this case, however, the distinction may not be so simple. There is a progestational compound now available for oral contraception, medroxyprogesterone acetate. This drug clearly affected glucose tolerance, particularly in diabetics and potential diabetics. (Vecchio, 1973). Moreover, the state of "insulin resistance" was more pronounced in patients taking chlormadinone, a progesterone derivative, than that seen in those treated with a combination or sequential type of oral contraceptive (Vermeulen et al., 1970).

Two pituitary hormones, vasopressin and oxytocin, cause hyperglycemia in animals as well as in man. They must be injected intravenously at a rapid rate to have the metabolic effect. Vasopressin requires the presence of an intact pituitary-adrenal system; oxytocin can cause hyperglycemia without such a functional system. The mechanism of action has so far not been determined (Baïsset and Montastruc, 1969).

There are still a few more mechanisms by which drugs can cause hyperglycemia. An interesting one is decrease in insulin synthesis. This is what the antitumor agent L-asparaginase is supposed to do. In leukemic patients treated with this drug, hyperglycemia is frequent and often very severe. Serum insulin levels are low and glucose tolerance impaired (Whitecar et al., 1970). In the presence of L-asparaginase insulin is fully active. This was not the case with N-monomethylacetamide, a substance which also caused hyperglycemia. Further analysis indicated that the compound did not reduce insulin production but interfered with its effectiveness. This finding was made in rats where the compound was tested as a potential solvent for benzothiadiazine diuretics. The nature of the hyperglycemic effect prevented its further use in pharmaceutical preparations (Sitt et al., 1966).

There are a few compounds which induce hyperglycemia, probably by an inhibition of membrane transport of D-glucose. This mechanism was suggested for the powerful hyperglycemic effect of i.p. injected 5-thio-D-glucopyranose in rats (Hoffman and Whistler, 1968) and the rather modest increase of blood glucose after chlorpromazine administration to the same animal species (Miya and Ghafghazi, 1968).

Finally, a direct toxic effect on the islets of Langerhans must be considered. As a recent example for such a compound, the antitumor antibiotic streptozotocin shall be mentioned. This drug has a high affinity for the β cells. After i.v. injection into mice and rats, insulin levels in the serum rise rapidly and hypoglycemia develops. But as insulin stores are exhausted, serum insulin levels decrease and hyperglycemia is established. Various degenerative changes and necrosis of the β cells can be observed microscopically (Junod et al.,

1969, Findlay et al., 1973). For those who must always see the finest and the earliest changes in a cell which is about to die, I recommend the paper of Lazarus and Shapiro (1972) who documented this agonizing process with excellent electronmicroscopic pictures. If one looks at an organ as closely as these authors did, one often finds more than originally expected. This was true in this instance since their rabbits also exhibited many degenerative changes in part of the α cells of the pancreatic islets.

Streptozotocin also affected the islet cells in man as demonstrated in several cancer patients. At the conventional dose of 1 - 2 g/m^2 given weekly by slow infusion, elevation of fasting blood sugar and disturbance of oral glucose tolerance was frequently observed. In some patients with insulin producing tumors, the treatment dramatically reduced serum insulin levels and prevented the dangerous hypoglycemia (du Priest et al., 1972). Thus, it is probable that the antibiotic destroyed β cells in man just as it did in animal experiments.

At this point a toxicological curiosity must be mentioned: there are a few compounds that produce reversible cytoplasmatic vacuolization of the β cells of the pancreatic islets in rats. With some of these substances fasting plasma glucose is elevated and glucose tolerance is reduced. This observation created some agitation among toxicologists, since it involved cyproheptadine, a drug widely used for appetite stimulation.

To put the clinician's and drug regulator's mind at ease, let me point out that the effect occurs only in rats and that sales of cyproheptadine in the rat population are negligible. In human patients the therapeutically effective doses of cyproheptadine did not alter serum insulin, fasting blood glucose, and oral glucose tolerance (Stiel et al., 1970, Lavenstein et al., 1962). That brings the problem way down on our worry-scale but high up on the scientific priority list. The fact that the dramatic morphological effect on the β cells cannot be produced in mice, hamsters, rabbits, dogs, and monkeys but readily in rats is unique. There are other features which make the observation an attractive object for biochemical toxicologists. How far they have gone to solve the problem is found in an up-to-date review presented by Fisher and Rickert (1975).

α cells, too, can be selectively damaged. The compound is neutral red, and its immediate effects after i.v. injection in rats were increased serum glucagon levels and hyperglycemia. α cells exhibited degenerative changes; in later stages they were completely destroyed, glucagon levels in the serum went down and the hyperglycemia disappeared. (Nevis et al., 1968). When serum glucagon was high and blood sugar markedly elevated, why did the β cells not respond as they should with immediate release of insulin? The experiments of Nevis et al., (1968) did indeed demonstrate such a response, but it occurred with a significant delay. This was explained by an epinephrine release following the dramatic appearance of glucagon from damaged α cells. Epinephrine, as was shown before, inhibits insulin release and thus, most probably had something to do with the delayed β cell response after injection of neutral red. With this finding we have come back full circle to where this review on drug-induced hyperglycemia has started: the metabolic changes mediated through catecholamines.

Clinical Significance

If one gives to an animal enough of just about anything, including a barrel of dirt, one can induce almost all toxic symptoms and all imaginable organ damages. That is what some unenthusiastic toxicologists keep saying. One wonders for what purpose such statements are made. Apart from the fact that the above generalization does not come even near to the truth, it certainly is of no consequence as far as society's legitimate demands for proper animal testing of new drugs are concerned. The symptom which I have selected for this discussion, hyperglycemia, is a good example to show what toxicological testing is all about. True, hyperglycemia can be induced with many substances, though not with all; true also, many of the effects reported in animals are of no consequence for the use of the drug in man. But others, perhaps only a few, are dangerous at least to some patients. And if the work with animals has helped to make present and future drug therapy just a little bit safer, the effort was justified.

Drug-induced destruction of β cells, such as was shown to occur with streptozotocin, is, of course, a serious finding which greatly reduces the usefulness of a new agent. The risk can be taken in cancer patients where the potential benefit is substantial and where consequent monitoring of glucose levels must detect early changes. This decision is made easier by the observation that β cell damage, at least in animals, is reversible (Findlay et al., 1973). Similar considerations should govern the application of L-asparaginase. The use of this drug presents a substantial risk as demonstrated by one patient who died in a hyperglycemic coma (Whitecar et al., 1970). But here again the severity of the disease and the potential therapeutic benefit often justify the risk. Monitoring of blood glucose is important, and if the levels are excessive or if signs of pancreatitis develop (high serum amylase), the drug must be withdrawn and restitution of pancreatic function must be awaited (Jaffe et al., 1972). Furthermore, one must know that the L-asparaginase effect may be potentiated by another diabetogenic drug such as prednisolone (Khan et al., 1970).

The clinical significance of hyperglycemia induced by the large group of drugs affecting glucose mobilization, utilization, and transport, insulin release, gluconeogenesis, etc., is not easily understood. Under clinical conditions, particularly in metabolically healthy subjects, significant disturbances of glucose metabolism are rarely seen. More severe changes occur on a "terrain diabêtique" to use the poetic description of a French colleague (Barjon et al., 1973). To use the prosaic American, we can state that substances such as diuretics, glucocorticosteroids, diazoxide and diphenylhydantoin may present a certain risk under the condition of "reduced pancreatic reserve" (Boyer, 1967). This means, first of all, that diabetic patients are most likely to be affected by these compounds. The next group includes the prediabetics and probably also healthy subjects with a family history of diabetes.

The most serious clinical consequence of a drug-related derangement of glucose metabolism is the hyperosmolar, hyperglycemic, nonketotic diabetic coma. This is a frequently lethal condition characterized by extreme hyperglycemia, glycosuria, and osmotic diuresis. This leads to dehydration often with hyperosmolarity, precoma, and ultimately, circulatory failure, epileptic convulsions, coma, and death (Evans et

al., 1972). This situation can also develop in metabolically normal individuals, particularly in patients where drug-induced disturbance of glucose metabolism is superimposed on a severe stress, e.g. infection, burns, pancreatitis, operations, hemolytic anemia, myocardial infarct, etc. (Boyer, 1967, Evans et al., 1972, Goldberg and Sanbar, 1969, Pierce and O'Brien, 1969). Stress, as I have related, has at least a dual effect on glucose metabolism leading to activation of glycolysis and inhibition of insulin release. The use of drugs, such as corticosteroids, adrenergic stimulants, diuretics, hypotensives, and diphenylhydantoin, in such severely ill patients may be the straw that breaks the camel's back; the agent that pushes blood glucose beyond the reach of all regulatory mechanisms.

It must be pointed out that the hyperosmolar, hyperglycemic, nonketotic diabetic coma is a rare complication. This proves that our physiological systems in control of glucose homeostasis are efficient. But sometimes a summation of extraneous influences, including drugs, can lead to severe damage. The physician must therefore be aware of the symptomatology of impending metabolic failure and know the drugs as well as the other circumstances which may precipitate the derangement.

Although I do not like to stir-up belligerent controversy, I must mention the possibility that "diabetogenic drugs" taken for prolonged periods of time might induce irreversible diabetes in metabolically healthy individuals. This suspicion is based on retrospective clinical observations which are, of course, always difficult to evaluate. A case in point is the suspicion that phenothiazines may induce diabetes. It was voiced by Thonnard-Neumann (1968) who observed a marked increase in incidence of genuine diabetes in hospitalized female psychotics in the years after introduction of chronic phenothiazine treatment. The author admits that many other factors, the diseases, the selection of the patient groups, obesity, more sophisticated diagnostic procedure, etc., could have influenced the statistical occurrence of diabetes in his hospital population. Thus, only a suspicion remains; an important problem for further clinical investigations.

Fortunately, drug-induced hyperglycemia occurring after the use of corticosteroids, diuretics, adrenergic stimulants, diphenylhydantoin, etc., is usually shortlived and of no serious clinical significance. The symptomatology may sometimes mislead a physician to a wrong diagnosis of diabetes. Klein (1966) reports such a case, a 20-month-old infant who was given generous helpings of an elixir against convulsions occurring during an acute febrile infection. The elixir had been dispensed by a pharmacist for another occasion. The physician, not knowing that it contained diphenylhydantoin, diagnosed acute diabetes and treated the hyperglycemia with insulin, achieving a very prompt and permanent response. The same almost happened to Baker et al. (1966) who saw three infants with hyperglycemia, glycosuria, and acetonemia. Theirs was also a pseudodiabetes, most probably due to intensive treatment of a cough with phenylephrine-containing medicine. They considered the diagnosis of diabetes but refrained from using insulin, and the condition cleared spontaneously.

Drug-induced inhibition of insulin release can obscure the symptomatology of an insulin-secreting tumor. Such a case was reported by Knopp et al. (1972) : A 44-year-old man, 17 years on antiepileptic therapy, was admitted to a hospital for operation of a phimosis. How much laboratory testing is needed before this intervention? Be this as

it may, a lowish fasting blood sugar on two out of three occasions was found and must have caught the eye of an alert physician. It triggered an extensive series of provocative challenges of the islet cell function with equivocal results at best. But when the tests were repeated after withdrawal of all medication it looked more like an insulin-secreting tumor. The islet cell tumor of considerable size found at the operation confirmed the difficult diagnosis. The patient left the hospital with only a small part of his pancreas left. Whether or not his phimosis was corrected, the authors do not relate.

And so it goes.

A word should be said about the significance of hyperglycemia and impaired glucose tolerance due to oral contraceptives. It is generally believed now that the effect is reversible. A retrospective study on 53 women who were the first to take these agents in clinical trials in Puerto Rico, some of them on drugs for as long as 183 cycles, gave no indication that diabetes might develop in metabolically healthy subjects. Only one patient on medication for 13 years became diabetic, but she had a family history of genetic susceptibility to the disease (Fuertes-De La Haba et al., 1971). This confirms the observation of others that in latent diabetics and prediabetics chronic administration of the hormones induces manifest diabetes (Terpstra et al., 1970). But several authors point out that even in diabetic women oral contraceptives are not necessarily contraindicated. There is clinical evidence that estrogens may improve maturity-onset diabetes (Javier et al., 1968), and a rather voluminous literature exists about equally beneficial effects of estrogens in various forms of experimental diabetes (Miya and Ghafghazi, 1968). It is thus perhaps reasonable to look at the disturbance of glucose metabolism in women on oral contraceptives as an indicator which must be kept under medical supervision so that a true diabetes could be recognized in time. That is one more reason why a woman who voluntarily (and not only for her own benefit) changes a hundred or more body functions with highly potent steroids must have these body functions checked regularly by a physician. Mail order medicine is not good enough for her.

VI

SYMPTOMATIC TOXICOLOGY

PSEUDOLUPUS

A New Disease

Lupus erythematosus is a serious, polysymptomatic disease of unknown origin. The sensational discovery of Dustan et al. (1954) and Perry and Schroeder (1954) that hydralazine induced an almost identical syndrome, was therefore greeted with great expectations. Later it was recognized that patients on prolonged procainamide therapy developed lupus erythematosus even more frequently, and other drugs were found, e.g. isoniazid, penicillamine, chlorpromazine, hydantoins, and certain antibiotics, which were also occasionally associated with the syndrome (Blomgren, 1973). The most characteristic feature of idiopathic lupus erythematosus is the presence of antinuclear antibodies. The same antibodies were found in drug-induced lupus. It was hoped, therefore, that the drug reaction would provide the magic clue that would help to unveil the secret which surrounded the strange collagen disease. But before we have deciphered lupus we are confronted with pseudolupus, a disease known only since 1972 (Maas et al., 1972).

As far as the symptoms are concerned, pseudolupus does not offer anything new. Patients complain of chronic, recurrent fevers lasting for days and weeks, and report all kind of aches and pains. Objective symptoms include arthritis, plauritis, pleural effusions, peri- and myocarditis, bronchopneumonia, and pulmonary fibrosis (Maas et al., 1972, Maas and Schubothe, 1973). Only very few cases of polyadenopathy and kidney involvement have so far been reported (Maintz et al., 1975). Typical laboratory findings include markedly accelerated erythrocyte sedimentation rate, absolute lymphopenia with normal or elevated white cell counts, and dysproteinemia. Antinuclear antibodies are absent, but all cases show antimitochondrial antibodies in high titres. This latter finding distinguishes pseudolupus from lupus erythematosus where antinuclear antibodies are consistently found. Antimitochondrial antibodies are rarely present in patients with lupus erythematosus (Maintz et al., 1975).

Drug-Induced Pseudolupus

Whenever a new drug is introduced there is a period of tense anticipation when you expect bad tidings almost around the clock. As the weeks and months pass you begin to relax, and after a few trouble-free years your confidence is so strong that you automatically blot out any suspicion that your compound might surprise you with unexpected toxicity. It must, therefore, have come as a shock to Grob and his collaborators when it dawned upon them that an old, popular and apparently harmless drug might have caused pseudolupus in several of their patients. The drug was Venocuran[R], in Germany also named Veropyronum[R], a combination containing 200 mg phenopyrazone, 20 mg horse-chestnut extract, and 2.5 mg glycosides extracted from Scilla maritima, Nerium oleander, Convallaria majalis, and Adonis vernalis. (Müller-Schoop et al., 1975, Grob et al., 1975). This compound is used quite widely for the treatment of venous diseases but is not sold in English-speaking countries.

The procedure used to corroborate the suspicion was as expedient as it was thrilling. First, Grob and his collaborators (1975) went through the list of patients with positive antimitochondrial antibodies. Of 68 there were 30 with chronic aggressive hepatitis and 26 with primary biliary cirrhosis. The remaining 12 had unspecified systemic disease, and of these 7 had no antinuclear antibodies. It turned out that all 7 had venous disease being treated with Venocuran[R].

Step 2 was to go hunting or begging for patients on long term Venocuran[R] therapy. The result was: 15 patients with the full pseudolupus syndrome, all showing high titre antimitochondrial antibodies, 10 users of the drug which were oligosymptomatic, of whom 9 had antimitochondrial antibodies, and 19 asymptomatic patients with antimitochondrial antibodies demonstrated in 14. No antibodies were found in 22 controls suffering from venous disease but undergoing a different mode of treatment.

Step 3 was to alert other investigators who promptly came forth with confirmatory evidence. Maas et al. (1975), the "inventors" of pseudolupus, found a clear-cut relationship between the drug and the disease in 45 of their 55 patients, and only 3 patients never had any contact with the combination product. Maintz et al. (1975), and Schwarz and Jost (1975) contributed additional 23 pseudolupus cases of which 19 had been exposed to the drug. Only in very rare instances were other drugs potentially involved.

Immunological studies with these patients gave essentially negative results. There were no antibodies against cell nuclei, native DNA, smooth muscle, erythrocytes, thyreoglobulin, and cytomegaly virus; antistreptolysine titres were not or only rarely elevated, rheuma factors and hepatitis B antigen were absent. Serological reactions for lues which are occasionally positive in idiopathic and hydralazine-induced lupus erythematosus were always negative.

After discontinuation of Venocuran[R] therapy patients improved slowly, but antimitochondrial antibodies often persisted. The same observation was made in hydralazine-induced lupus erythematosus where antinuclear antibodies often remain for many years, although the symptoms have completely regressed.

Although a causal relationship between chronic intake of Venocuran[R] and pseudolupus seems highly likely, there remain many open questions: For example, which of the components of the product is to blame? The pyrazolone derivative is used in an antipyretic compound which has, so far, not been associated with the pseudolupus syndrome. Horse-chestnut extract occurs in many preparations prescribed for venous disease, and the mixture of glycosides is sold as a mild cardiac stimulant, apparently also without problems (Grob et al., 1975).

Venocuran[R] itself has been on the market for 15 years, but only very few cases of pseudolupus were known up to a few months ago. It seems unlikely that this serious disease would have been overlooked for more than 10 years. I speculate, and I have nothing to go upon other than my suspicion and the memory of a similar experience, that a new and toxic contaminant may have been introduced into the product, be it by a change of the manufacturing process or a new source of raw material. Unless an animal assay system for the toxic effect can be developed it will be impossible to settle this question.

An even more important problem pertains to the mechanism of action of the toxic component(s). Is pseudolupus a hypersensitivity reaction or a cumulative toxic effect? We remember that the same question was asked when the hydralazine syndrome was discovered (Reynolds and Caldwell, 1957). But I don't recall that it has ever been answered convincingly. The fact that almost 90% of the patients who took Venocuran[R] for a prolonged period of time developed antimitochondrial antibodies, even if they had no systemic symptoms (Grob et al., 1975), is difficult to reconcile with an allergic process. Moreover, the observation that a prolonged period of treatment is necessary before the disease becomes manifest is rather in keeping with a cumulative toxic effect. Animal experiments which are certainly now underway in many places may give us an answer.

Toxicological Significance of Antimitochondrial Antibodies

The unique aspect of the pseudolupus syndrome is the presence of antimitochondrial antibodies which are found in 100% of the patients and, apparently, also in the majority of the users of Venocuran[R] who do not exhibit any symptoms of the disease (Grob et al., 1975). Up to now these antibodies were considered to be very characteristic for a few liver diseases, mostly primary biliary cirrhosis. In this rare condition they are present in over 90% of the patients and are directed against the inner membrane of the mitochondria (Berg et al., 1972). Serologically they may be distinguished from the antibodies occurring in pseudolupus (Berg et al., 1975). In non-hepatic diseases antimitochondrial antibodies are rarely found (Berg et al., 1973).

The presence of these antibodies by itself doses not seem to be anything special. Immunologists expect them whenever tissue damage occurs, be it as a consequence of a virus infection or even after injuries and necrosis. In rats, for example, a small dose of carbon tetrachloride (1 - 2 x 0.3 ml/kg) was followed by an increase in antibodies (Pinckard and Weir, 1966) which were directed mostly against the outer mitochondrial membrane. The effect was, however, of short duration and disappeared after 7 days (DeHeer et al., 1972). Just in case

you also wish to look for such antibodies in the serum of your rats, note that the antibodies may occur naturally. De Heer et al. (1972) observed antibodies against whole mitochondria in 21% of their rats, antibodies against the inner mitochondrial membrane in 14%, and against the outer membrane in 36%. This finding is explained by the inability of the rat to develop immunological tolerance against various subcellular constituents.

In the normal human population antimitochondrial antibodies are found in less than 1%. They do occur, however, in drug-induced jaundice. For example, Rodriguez et al. (1969) demonstrated them in 7 of 9 cases with liver toxicity following halothane narcosis, and in 4 of 7 instances of obstructive jaundice after chlorpromazine therapy. There were no antimitochondrial antibodies in 3 cases of jaundice due to methyldopa and 1 case each of hepatotoxicity induced by aminosalicylic acid and chlorpropamide. The number of such observations is, however, too small to decide whether or not the demonstration of antimitochondrial antibodies will provide useful information about cause, type and prognosis of drug-induced hepatic damage. But it is important to note that antimitochondrial antibodies were not observed in 10 patients each treated with halothane or chlorpromazine without developing symptoms of hepatotoxicity. It is possible, therefore, that the antibodies occurring in the jaundiced patients are nothing but a normal immunological reaction of the organism against the release of subcellular particles.

The toxicological significance of the antimitochondrial antibodies after chronic administration of Venocuran^R, on the other hand, lies in the observation of Grob et al. (1975) that even patients without any disease symptoms frequently showed a positive immunological reaction. In general the titres were higher in patients treated for a longer period of time. We could thus look at this laboratory finding as an early warning symptom which becomes an important diagnostic tool for the identification of other substances which might also have a potential for causing the pseudolupus syndrome.

HEALTH HAZARDS FROM COLORS

Magnitude of the Problem

Like politicians of all shades, food, drug, and cosmetic colors have a high visibility. This explains why there is more public concern about them than they truly deserve. Some colleagues, however, believe that we do not worry enough about colors, and they reinforce their contention with impressive statistics. Did you know, for example, that the total production of primary colors used in foods in the United States during the first 9 months of 1967 was 1,916,179 lbs? Well, deduct the 202,565 lbs. which went into pet foods, and the 2,271 lbs. used to stamp meat, but we are still left with an impressive figure. Think also of the 72,156 lbs. used in pharmaceuticals, and you end up with a lot of colors devoured by the American consumer.

People feel instinctively threatened by large figures regardless of what they imply for the individual. People also resent the constraint to consume colors which manufacturers add to their convenience foods. And don't try to explain that it is much more dangerous to swig a double martini and to smoke a pack of cigarettes than to eat a bowl of rainbow-colored cereals. They will just not listen to such arguments. As Handler (1974) recently said: ..."we must acknowledge the difference between publicly inflicted and privately accepted risk." Doctor Handler has estimated that ..."most of us will voluntarily accept risks about two orders of magnitude greater than we will accept when the rest of society imposes them."

People also feel uneasy if they are confronted with a threat whose order of magnitude they cannot judge. You don't have to scare them with the old stories about the London caterer who served a green pudding at a public dinner, and caused the death of two customers because the green color was copper arsenite, or about the lady who, in 1820, paid with her life for the bad habit of eating pickles while at her hairdressers, that is pickles which were colored green with copper sulfate (National Academy of Sciences, 1971). No, it is enough to imply that we really don't know enough about the safety of today's food colors, for example by writing a sentence like this: "Regardless of the economic or mental standards of our population, every individual ingests some quantity of food dyes which may or may not have deleterious physical effects." (Hosen, 1972).

Feelings of uneasiness and fear pile up and result in a neurosis which you can turn into a profit by writing a book or a magazine article about it. Other troubled souls will learn from you and add food, drug, and cosmetic colors to their other environmental worries, such as antibiotics residues, pesticides, radioactive fallout, hormones, bacteria, cholesterol, air pollution, and the fading ozon layer in the skies. Since these environmental impacts are, without exception, potential causes of disease, it is quite understandable that you will ask your doctor about them when you see him at the next cocktail party. Not many physicians are, I am afraid, familiar with the present state of environmental toxicology. It is thus for my medical friends that I present these few aspects of color additive toxicology.

Some Facts about Color Additives

If, at your next cocktail party, someone shows you a bright red Maraschino cherry in his Manhattan and complains about the innumberable artificial colors contained in our foods, let him guess how many dyes there are which may be added to foods. The number varies from country to country. Australia permits 7 synthetic and 6 natural dyes, the United Kingdom 22 and 23, respectively. The other countries are somewhere in between the conservative Australians and the liberal British.

Tell your friend also that the use of the permitted colors is limited by laws and regulations. And if he doubts this statement inform him that eating his Maraschino cherry is the only opportunity of an American citizen to consume legally F D and C Red No. 4. If it is Orange B he wants to try, he must eat it with a Frankfurter or another sausage. And there is no other way to get it.

The discussion will probably turn now to the monumental question how the (expletive deleted) government knows how much of these colors he can tolerate. This is your opportunity to tell him about the ADI, the acceptable daily intake, which is determined from animal experiments and other safety data. The ADI is the amount of a color which a group of rather conservative scientific experts believes can be ingested daily, even over a lifetime, without risk.

One of these expert groups which is not only conservative from the scientific point of view, but must also accommodate divergent opinions in countries around the globe, is the Joint FAO/WHO Expert Committee on Food Additives. On its list (shown in Table 8) we find 7 synthetic and 11 natural colors. Note that some of the natural colors, the carotenoids, are now also produced synthetically.

Toxicological studies with food colors, particularly the ones produced synthetically, far exceed those customarily available with other substances, including drugs, pesticides, cosmetics, common foods, and industrial chemicals. So if somebody implies that we really don't know much about the biological effects of the colors in a lollipop or in an ice cream cone, you can safely state that more extensive toxicity testing was performed with the colors than with all the substances accounting for the remaining 99.99% of the sweets.

TABLE 8

Acceptable Daily Intake of Food Colors, as Recommended by the Joint FAO/WHO Expert Committee on Food Additives (1974)

Synthetic Colors	ADI (mg/kg)	Special Remarks
Amaranth [1)]	0 - 0.75	Temporary. Requested: completion of additional toxicity studies, particularly teratology and carcinogenicity
Azorubine, Carmoisine	0 - 0.5	Temporary. Requested: completion of additional toxicity studies
Brilliant black PH	0 - 2.5	Temporary. Requested: completion of metabolism and reproduction studies
Erythrosine	0 - 2.5	
Indigotine (Indigo Carmine)	0 - 5.0	
Ponceau 4R	0 - 0.125	Temporary. Requested: completion of long-term study in 2nd species, metabolism and reproduction studies
Quinoline yellow	0 - 0.5	Temporary. Requested: completion of long-term study in 2nd species, metabolism and multigeneration studies
Natural Colors		
Annatto extracts	0 - 1.25	Temporary. Requested: completion of metabolism studies
Beet red	not specified[2)]	Temporary. This normal food constituent has not yet had the benefit of adequate animal toxicity testing
Canthaxanthine	0 - 25.0	
Caramel colors (ammonia process)	0 - 100.0	Temporary. Requested: resolution of problem regarding various imidazoles and pyrazines developing during manufacture

β-apo-8'-carotenal	0 - 5.0[3]	
β-carotene	0 - 5.0[3]	
β-apo-8'-carotenoic acid methyl and ethyl esters	0 - 5.0[3]	
Chlorophyllin, copper complex	0 - 15.0	
Curcumin	0 - 0.1	Temporary. Requested: completion of additional toxicity studies
Turmeric	0 - 2.5	Temporary. Requested: completion of additional toxicity studies
Iron oxides	not specified[2]	

1) not yet published

2) does not appear to present a hazard. The Committee does not allocate an ADI

3) expressed as sum of total carotenoids

Food colors constitute a small portion of our daily diet. In the so-called convenience foods their concentration rarely exceeds 400 ppm. Colored beverages contain about 75 ppm. The average daily consumption of food colors in the United States was calculated to be approximately 15 mg. These figures were arrived at from the average amount of food ingested per capita. Were one to select always the foods that contain the highest color concentration one would, according to the same calculations, receive slightly over 50 mg of dyes per day (National Academy of Sciences, 1971).

Toxicological guidelines require that substances must be given to animals at various dose levels, one of which must cause toxic effects (ST I, 4 - 17). This is difficult to do with food colors, since most of them are quite inert. In rat experiments you often have to go as high as 5 or even 10% of the dye in the food. An eye witness who went to Japan to observe toxicity studies in progress with Violet No. 1 reported that not only the rats, but also the cages, the animal caretakers and the whole research building were colored violet.

Most of the colors used in foods, drugs, and cosmetics have been around for quite some time. It is inevitable, therefore, that many safety studies performed with them do not conform anymore to modern standards. Moreover, new toxicological concepts are elaborated continuously. For this reason regulatory agencies and expert groups request that safety studies with the permitted colors must periodically be reviewed and, if necessary, upgraded. This explains why more than half of the colors on the permitted list of FAO/WHO have only a temporary ADI (Table 8). The additional studies required reflect the Committee's current interest in potentially toxic metabolites, the effects on fertility, reproduction, and fetal development, and in potential interactions

with other food constituents. Future studies may deal with changes of the additives during food processing, sterilization, storage, and spoilage.

All these investigations are designed to strengthen our confidence in the harmlessness of these widely distributed substances. But will we ever be sure that they are totally safe? Can we prove the absence of all toxic effects? The experts recognize that this is not possible. All we can do is to search continuously and intensively for undesired effects; we must look, as my son's drawing on page 88 illustrates, for the hidden needle in the haystack. It is not as tedious a job as it seems from the faces of the toxicologists in the picture. On the contrary, it is interesting and challenging; it might even be quite enjoyable, were it not for the nameless fellow on the left side of the picture who keeps telling us how to go about our work. Color him (Butter) yellow!

Medical Problems with Colors: Carcinogenicity

Compared to the toxicologists', the physicians' troubles with colors in foods and drugs have been few. Their major concern, of course, has been the potential carcinogenicity of these foreign substances. Textile colors were among the first triumphs of the chemical revolution, and many of them were indiscriminately chosen as food additives. This practice, however, was swiftly abandoned when bladder cancer was shown to be induced in man by aniline dyes. Extensive testing of azodyes disclosed that many caused tumors in animals, the most widely cited example being Butter Yellow (Hecht and Wingler, 1952). For the record, however, it should be noted that this compound was withdrawn from the US market in 1918, 19 years before its carcinogenic action in rats was discovered, the reason being a high incidence of contact dermatitis in workers engaged in its manufacture (National Academy of Sciences, 1971).

Physicians must know that presently available food colors are extensively tested for carcinogenicity. The evaluation of these experiments is based on the concept that "any substance which produces cancer in any species of animals at any dosage level should not be allowed in human food." (Boyland, 1958). You may therefore assure your troubled patients that food colors are safe.

So far so good. But can you now spare a few minutes to listen to the troubled toxicologists, so that they may tell you about their problems with food additive carcinogenicity? Their major difficulty is the fact that the idealistic concept as expressed in the above cited statement of Boyland (1958) is now chipped in granite in the form of the famous Delaney clause of the Federal Food, Drug, and Cosmetic Act. How would it sound to you as a surgeon if a law would proclaim "an operation shall be deemed unsafe, and shall not be performed, if the operation is found by the Secretary to introduce pathogenic bacteria into man or animal ..." It sounds silly, doesn't it? And this is the way the Delaney clause for food color reads:

"Sec. 706 (b) (5) (B) A color additive (i) shall be deemed unsafe, and shall not be listed, for any use which will or may result in ingestion

of all or part of such additive, if the additive is found by the Secretary to induce cancer when ingested by man or animal, or if it is found by the Secretary, after tests which are appropriate for the evaluation of the safety of additives for use in food, to induce cancer in man or animal, and (ii) shall be deemed unsafe, and shall not be listed, for any use which will not result in ingestion of any part of such additive, if, after tests which are appropriate for the evaluation of the safety of additives for such use, or after other relevant exposure of man or animal to such additive, is found by the Secretary to induce cancer in man or animal ..."

There is a heated discussion among scientists in which many laymen have joined, about the justification of this clause. Many believe with Boyland (1958) that "The principle that there is no safe dose of any carcinogen has been generally accepted in Europe as the basis for regulations governing the use of food additives." Others maintain that the law as it is written is unscientific: "The words of the Delaney clause allow no consideration of dose-response, as I read them. I am aware that there are others who believe that the principle of zero tolerance must be accepted for any substance shown in any concentration to be carcinogenic in animals. In their thinking, a safe level for man cannot be established for agents shown in any species to be carcinogenic. They do, however, recognize as a realistic notion the concept of, as they call it, 'socially acceptable risk.' That notion is foreclosed by the clause. The experimental feeding of high concentrations of a particular substance as a caricature of what man conceivably could ingest is toxicologically naive and also ignores basic pharmacologic principles. It is possible with high concentrations to overload the system of elimination by which a substance normally would be handled. Under those circumstances alternative pathways of elimination can be called into play or the substance can accumulate in an abnormal way. The alternate (unreal) pathway or the accumulation itself could be the factor implicated in the end result observed. An experiment of this nature bears little relationship to reality." (Wescoe, 1974).

These are only two of many opinions expressed with conviction for or against the Delaney clause. There is no telling about the ultimate outcome of this contest. What I can tell you, however, is how the clause works. I will illustrate it with an example of the last food color which was de-listed (that means, it was terminated with extreme prejudice), a few years ago. The victim was Violet No. 1, an ugly color used mostly to stamp meat. In a chronic feeding study 20 male and 20 female rats received 89.1% pure (that is poor quality) Violet No. 1 as a 5% admixture to the diet. Ten rats each of both sexes were used as controls. The experiment did not go well, most animals had pulmonary infections, and half of the treated rats were dead after 200 days. Only a very few survived for one year. None of the males showed tumors. Five treated female rats had mammary carcinoma, and 4 had ear duct carcinoma. One female control showed a fibroadenoma of the mammary gland. The authors, Uematsu and Miyaji (1973) mention another Japanese study which also indicated carcinogenic potential of the dye. Based on this information the American regulatory agency made use of the Delaney clause and banned the dye. It also initiated substantial confirmatory animal studies.

The question whether this was an inevitable or an inconsiderate reaction by the agency to an inconclusive animal experiment is still exctiting the scholars. It did receive - and still does - broad coverage

in the scientific and lay press. The decision was of little practical importance and certainly did not influence the American cancer statistics. But it brought into the open a widespread uneasiness about the way scientific matters are influenced by legal considerations.

By coincidence, the same issue of the Journal of the National Cancer Institute that carried the report about the rat carcinogenicity experiment with Violet No. 1 also contained a paper by Grundy and Uzman (1973) which draws attetnion to a serious health problem from multiple fluoroscopy. It confirms a previous report by Myrden and Hiltz (1969) who observed an alarming breast cancer incidence of 7.3% in 300 tuberculous women who received multiple fluoroscopy for control of pneumothorax. Significantly, most breast cancers were located on the side of the pneumothorax. Note, that these were people,mostly women of the younger age group, who got cancer,and not mice or rats. But there was much less public concern and press coverage of this serious health hazard than for Violet No. 1, and no regulation was passed to control the indiscriminate use of fluoroscopy.

Coming back now to the toxicological aspects of the carcinogenicity problem, let us consider briefly the controversy about the significance of certain animal tests. The most disputed experiments are those in which a carcinogenic effect - an increase in tumor incidence or an earlier appearance of tumors - occurs only if small rodents are treated for a lifetime with excessively high doses. The proponents of the Delaney clause are convinced that even such a result is relevant for man. Their opponents believe that overloading of excretory and metabolic systems leads to an unrealistic accumulation of the test substances and this may contribute to the development of tumors. They predict that these tumors would not occur if the animals were exposed to concentrations as low as those reached in man. Neither side has as yet proven its case, but so far, it seems to me that the NNELOAC (no no-effect level of any carcinogen) party has presented the more convincing arguments.

If we want to criticize unrealistic experiments we should not be contented with general terms such as "overloading of excretory mechanisms." We need better evidence such as that presented in a recent and fascinating paper of Riley (1975). The author demonstrated a highly significant delay in the appearance of mammary tumors in C 3H/He mice which were carefully protected against environmental stresses such as noise, frequent handling, poor housing conditions, and drawings of blood samples. As a possible explanation we are reminded of the consequences of stress: increase in plasma corticosterone levels, fall in thymus-dependent T lymphocytes, and thymus atrophy. This could lead, so the author speculates, to a breakdown of the immunological surveillance system which is thought to eliminate transformed malignant cells. A similar weakening of the resistance against the development of (virus induced?) tumors, might conceivably also play a role in carcinogenicity tests during which the animals suffer from grotesque overdosage with harmless substances.

Medical Problem No. 2: Hypersensitivity

A few azo dyes commonly used in foods, drugs, and cosmetics have been implicated as causes of allergic reactions. Clinically the symptoms were either related to the respiratory system - vasomotor rhinitis, chronic nasal blockade, nasal polyps, even bronchial asthma - or to the skin where the reaction appeared as angioneurotic edema or recurrent urticaria. In order to prove a link between these allergies and colors in the environment you have to be a tenaceous and courageous physician.

You have to be tenaceous because it takes much time and persuasion to get your patient (a) to refrain from eating food that neither contains artificial additives nor naturally occurring benzoates and salicylates, (b) to be blindfolded while swallowing a capsule with a small amount of a food color (Michaelsson and Juhlin, 1973) or to have the lousy tasting stuff applied sublingually (Lockey, 1973), (c) to wait for 30 minutes for an allergic reaction to happen, and (d) to go through with the whole procedure again and again until the allergen(s) is (are) apprehended. You also must be courageous, because this procedure (I now let somebody talk who does such investigations frequently) "...requires anticipation of severe angioedema, bronchial asthma, cyanosis, asphyxia, and possible coma, and should not be undertaken unless an anesthesia team is present to maintain open airways if necessary." (Samter and Beers, 1968).

Of the azodyes known to cause the hypersensitivity reactions probably the most active one is Tartrazine (also designated as FD & C yellow No. 5, CI Acid Yellow 23, C.I. 19140, hydrazine yellow, CI Food yellow 4, Lebensmittelgelb No. 2) (Cohon, 1975). Others which are only rarely implicated are Amaranth (FD & C red No. 2), Sunset yellow (FD & C yellow No. 6), Brilliant Blue FCF (FD & C blue No. 1), Ponceau SX (FD & C red No. 4), and New Coccine (Chafee and Settipane, 1967, Michaelsson and Juhlin, 1973, Fisherman and Cohen, 1973, Hubbard, 1974).

Since these colors are used in many products - Cohon (1975) has identified over 600 drugs that contain Tartazine - it is almost impossible for an allergic patient to escape contact with such allergens. Moreover, most patients who are sensitive against one of these colors also react to other substances. For example, in a group of 39 patients suffering from recurrent urticaria, 35 reacted to aspirin, 22 to Na benzoate, 21 to OH-benzoic acid, 19 to Tartrazine, 10 to Sunset yellow, and 9 to New Coccine (Michaelsson and Juhlin, 1973). Similar results were reported by Samter and Beers (1968). They tested 182 patients who were allergic to aspirin (respiratory symptoms 154, skin symptoms 18, and both manifestations 10). Forty were allergic to various drugs, 26 to foods, 14 to alcoholic beverages, 14 to Tartrazine, 12 to perfumes, and 10 to environmental inhalants.

These are studies with a highly selected patient population whose major problem was allergy to aspirin. It is interesting to note that many patients did not realize that their disease was due to the pain reliever. I doubt that any of them even considered that a food color, a food additive or a naturally occurring salicylate or benzoic acid derivative might also have something to do with their persistent health problem.

In the normal population the number of patients who have been identified as allergic to food colors is very small; it does not justify an arduous campaign against these products. If you are, however, looking for a serious environmental factor and a worthy cause in which to get involved, read the paper Smith et al. (1969) wrote about their asthmatic children. By skin-testing 302 of them they found that 77% reacted positively against the house dust mite Dermatophagoides pteronyssinus. You will find this beast on the surface of our mattresses where it feeds on shed human skin scales. It and its tiny fecal pellets are highly allergenic and represent, at least in certain areas of England, the major cause of bronchial asthma. Unformtunately, we cannot de-list it or ban it with the help of a Delaney clause. Is that perhaps, the reason why we would rather turn our back upon Dermatophagoides pteronyssinus?

Finally: Hyperkinesis

When Dr. B.F. Feingold published his book "Why your Child is Hyperactive" (Random House, 1975) he became an instant success with many parents. Had we not all somehow suspected that our genes, our marital problems or our drinking habits may have something to do with little Johnny's troubles at school, his poor report card and his inability to sit still for more than 2 seconds? Now, the Doctor claims, it's because of his food, that is the artificial colors and flavors and the natural salicylates in his food. "One candy bar", he says, "loaded with additives can make a child hit the roof. It might take a week for him to come down." (New York Times, February 9, 1975). If this does not sound very scientific to you, note that Dr. Feingold does not claim to be a clinical pharmacologist. He is a 74 year old pediatrician who tries to explain why our children are much more hyperactive, and quite destructively so, than those of his generation. His suspicion of allergies to things which were not in foods when he, you and I were growing up, cannot be dismissed as totally unfounded. The possibility that allergic reactions may affect behavior must not be ruled out. Whether or not Dr. Feingold's miracle K.P. diet which contains no food additives and natural salicylates will cure my Johnny and your Billie from hyperkinesis cannot be predicted from existing data. There are, however, controlled experiments under way. I know of parents who pray that these studies will prove Dr. Feingold to be right.

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