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The Herpesviruses – A Biochemical Definition of the Group

Bernard Roizman

With 10 Figures

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I. Introduction

"When we give a definition it is for the purpose of using it".

HENRI POINCARE in *Science and Method*

A. Objectives

The first version of this paper was written to introduce new students and fellows of my laboratory to the mysteries of herpesviruses. Consonant with this design sections dealing with well documented data were trimmed to the bone whereas many obscure phenomena, controversial data and seemingly trivial observations were discussed generously and at length. There is some doubt as to whether it was meant to be published, but it was not a review. The objective of reviews is frequently to bring order. But alas, even the most fluent summation of credible data frequently makes dull reading and too much plausible order, like very little entropy in chemical reactions, is not the most suitable environment on which to nurture the urge to discover. This version is more charitable but not less inbalanced. The bibliography reflects the intent of the paper and was updated last in December of 1968. It should be obvious without saying that no single account such as this can do justice or injustice, as the case may be, to the several hundred papers published on herpesviruses each year or to the many thousand papers published on herpesviruses since the first of the members of the family was experimentally transmitted to a heterologous host more than half a century ago (GRUTER, 1924).

B. Definition

1. Derivation of the Name

The word herpes is derived from ἕρπειν meaning to creep and recurs in medical texts dating back at least twenty-five centuries. In the Hippocratic Corpus the term was applied to spreading cutaneous lesions of varied etiology, usually ulcerative, severe and difficult to treat (BESWICK, 1962). In the intervening centuries the use of the term became restricted to certain diseases characterized by vessicular exanthema; herpes zoster, derived from ζωστρ a girdle, alone retained the name herpes throughout the centuries. A clear account of herpes labialis as a distinct clinical entity appeared in 1694 (BESWICK, 1962). Herpes catarrhalis, progenitalis, facialis and simplex appear in the 18th and 19th centuries (BESWICK, 1962). GRUTER (1924) was the first to report the successful transmission of herpesvirus from man to rabbit. In vivid contrast to the practices of today he did the work between 1911 and 1914 but did not publish until nearly 10 years later. The discovery of what appeared to be a single causative agent led to the belief despite objections by LIPSHUTZ (1921, 1932) that herpes genitalis, facialis and febrilis are all different clinical manifestations of one disease — herpes simplex. The virus took on the name of the disease. Nearly 30 years elapsed before it again became apparent that the waistline probably does separate herpes simplex viruses of man (and also

herpesviruses of cattle and horses!) into two groups with distinct biologic and chemical properties. A clear account of the early history of herpes simplex is given by NAHMIAS and DOWDLE (1968).

2. Classification

Herpesviruses are formally defined as large enveloped virions with an icosahedral capsid consisting of 162 capsomeres and arranged around a DNA core (LWOFF et al., 1962; LWOFF and TOURNIER, 1966). A few of the viruses usually included in the herpesvirus group (WILNER, 1966) are herpes simplex, B virus, marmoset virus, pseudorabies virus, equine herpesvirus, varicella-zoster virus, cytomegaloviruses (of man, guinea pig, mouse, swine, etc.), a number of bovine (lumpy skin disease, infectious rhinotracheitis and mammillitis viruses) canine, avian, rabbit, (virus III), and feline herpesviruses. Only common names are used in this chapter. The binomial name (ANDREWS, 1962), which contains in addition to the generic "Herpesvirus" the name of the species in which the virus is found, generously allows only one herpesvirus per host species even though man alone has at least three whereas horses and cattle probably do even better. The viruses meeting the structural and architectural criteria for inclusion into the herpesvirus group also share in common many unique features of their replicative processes. Among the most interesting aspirants for inclusion in the herpesvirus group is the virus found in cells from a human lymphoma (BURKITT, 1958) grown *in vitro*. The virus associated with the lymphoma meets some of the structural and biologic criteria for inclusion in the herpesvirus group (EPSTEIN et al., 1964; EPSTEIN and BARR, 1965; O'CONOR and RABSON, 1965; STEWART et al., 1965; HUMMELER, 1966; YAMAGADI et al., 1967). Another aspirant is a virus seen in cells grown *in vitro* from fowl afflicted with Marek's disease (WIGHT et al., 1967; EPSTEIN et al., 1968), but at the time this paper was written the nucleic acid of these two viruses had not been characterized. Another interesting candidate is a virus described by FAWCETT (1956) and by LUNGER (1964) as present in the nuclei of an adenocarcinoma (Lucké tumor) of the frog, Rana pipiens. The virus is probably oncogenic in that extracts of tumors containing this virus cause kidney tumors in metamorphosing frogs (TWEEDELL, 1967). The properties of the nucleic acid of the virus are at this time unknown. Structurally however, it resembles herpesviruses (LUNGER, 1964; STACKPOLE and MIZELL, 1968). The fly in the ointment, interestingly enough, is a group of viruses isolated from both healthy and tumor bearing frogs and grown in cultures of fathead minnow, chick embryo and in baby hamster kidney cells (GRANOFF et al., 1965; RAFFERTY, 1966, 1967; LEHANE et al., 1968). These viruses contain DNA (MORRIS et al., 1966) but multiply in the cytoplasm and acquire an envelope from the cytoplasmic membrane (DARLINGTON et al., 1966; MORRIS and ROIZMAN, 1967). The classification of these viruses is uncertain. It seems likely that the isolates are not herpesviruses but are members of a poorly defined group of DNA viruses comprising African horse sickness virus (LECATSAS and ERASMUS, 1967) and possibly tipula irridescent virus (ALMEIDA et al., 1967).

There have been suggestions (MELNICK et al., 1964) that herpesviruses should be segregated into two subgroups on the basis of certain chemical and biologic properties. Thus infectious herpes simplex and pseudorabies viruses are readily released and extracted from infected cells. The DNA of these viruses are characterized by a relatively high G + C content. As an example of the other subgroup, cytomegalo viruses have a DNA of a lower G + C content and are considerably less infectious when released or extracted from infected cells. The lack of infectivity of the cell-free virus may be a reflection of the instability or the lack of cohesiveness of the structural components of the virus outside the nucleus (COOK and STEVENS, 1968).

II. Fine Structure and Composition of the Herpesvirion

> "According to the Platonic concept, all things originate in their opposites. Darkness comes from light, cold from heat, pleasure from pain. Biological order had its origin in primitive disorder. But if the question is asked: what can originate from order: the only possible answer is disorder".
>
> ANDRE LWOFF in *Biological Order*

A. Architecture

1. Sources of Information, Definition, and General Description of the Virion

Information concerning the structure of herpesviruses is derived from three sources i.e., (i) thin sections embedded in a suitable plastic and stained with heavy metal salts, (ii) dried preparations of particles permeated with phosphotungstic or silicotungstic acid (negative stain techniques) and (iii) chemical and biologic studies of isolated components. Correlations among these techniques are difficult enough at best, but nearly impossible without an adequate terminology. For our purposes the terminology proposed by LWOFF et al. (1962) and LWOFF and TOURNIER (1966) is a useful starting point. There is general agreement that the herpesvirion consists of a core, a capsid and an envelope. Specifically, the *core* is defined as a centrally located body containing viral DNA and probably viral protein with particular affinity for DNA. The core is enclosed in a *capsid*. The *structural subunit* of most capsids of animal viruses consist of proteins. The structural subunit of most capsids of animal viruses consists of proteins, which may be associated in clusters or *capsomeres*. The envelopes of animal viruses, when present, consist of lipoproteins probably arranged in an orderly structure. The capsid and core form the nucleocapsid. The virion is defined as an enveloped nucleocapsid.

The literature on the structure of herpesviruses is very extensive; to avoid the morass of contradictions and terms that make sense only to their authors, it seems desirable to take an argumentative rather than a descriptive approach in the discussion of the fine structure of the virion. The available data seem to suggest that the structure of the virion consists of (i) a core 25—30 nm in diameter containing viral DNA and probably protein, (ii) an inner capsid 8—10 nm thick, (iii) a middle capsid 15 nm thick (iv) an outer capsid 12.5 nm

thick and consisting of 162 capsomeres, (v) an inner envelope 10 nm thick probably containing lipids and materials with low affinity for electron opaque heavy metal salts and lastly (vi) an outer envelope approximately 20 nm thick. The data supporting this hypothesis concerning the structure of the herpesvirion and some of the properties of the architectural components, follow.

2. The Number of Structural Components

The presence of at least 6 structural components in the virion is discerned from thin sections of infected cells. It is generally agreed that nucleocapsids approximately 100 nm in diameter either dispersed or in crystalline assays are a common feature of the nuclei of infected cells, whereas enveloped nucleocapsids or virions 150—170 nm in diameter are sequestered in structures delineated by membranes and projecting into the nucleus or, more frequently, into the cytoplasm. The electron photomicrographs reported by FELLUGA (1963), CARMICHAEL et al. (1965), RABIN et al. (1968), and by SIEGERT and FALKE (1966) show intranuclear particles 100 nm in diameter consisting of a semi-translucent center surrounded by 3 shells, an electron opaque, translucent, and opaque, in that order. In electron photomicrographs published by others (MORGAN et al., 1953, 1954, 1958, 1959; STOKER et al., 1958; FALKE et al., 1959; MORGAN and ROSE, 1960; EPSTEIN, 1962a, 1962b; WATSON et al., 1964; LUNGER, 1964; LUNGER et al., 1965; MCGAVRAN and SMITH, 1965; BECKER et al., 1965; SHIPKEY et al., 1967; NII et al., 1968a; SPRING et al., 1968) the semi-translucent center and the first electron opaque shell fuse into an electron opaque body. The mature virion as seen in thin sections contains two additional shells, the inner being electron translucent whereas the outer is electron opaque. EPSTEIN (1962a) and ZAMBERNARD and VATTER (1966) demonstrated by means of nuclease digestion that the electron opaque center of the virion contains DNA.

3. The Inner and Outer Envelopes

It is generally inferred that the envelope of the virus (henceforth designated as the outer envelope) is derived from the inner lamallae of the nuclear membrane (MORGAN et al., 1954; FALKE et al., 1959; SIEGERT and FALKE, 1966; FELLUGA, 1963; NII et al., 1968a). The intact outer envelope is impervious to phosphotungstic acid. The stain readily penetrates virions modified by drying or by exposure to antibody reactive with the envelope (WATSON, 1968). The thickness of the outer envelope is difficult to estimate since the envelope tends to collapse on drying and under centrifugal stress. We estimate it to be 20 nm thick (SPRING et al., 1968). It appears to consist of two layers each showing a repeating unit structure (Fig. 1 A). Occasionally spikes seem to project from the surface of the outer envelope; this happens particularly when excess negative stain fills the space between the repeating unit. We have the impression that the envelopes of virions freshly extracted from infected cells form a tight sheath around the nucleocapsids and do not collapse as readily as those of particles stored for several days.

The evidence of an inner envelope is based on two observations. First, in order to be enveloped, the nucleocapsid comes in opposition to the nuclear membrane where it is enfolded. Electron photomicrographs of thin sections show that the nucleocapsid maintains a fixed distance from the membrane

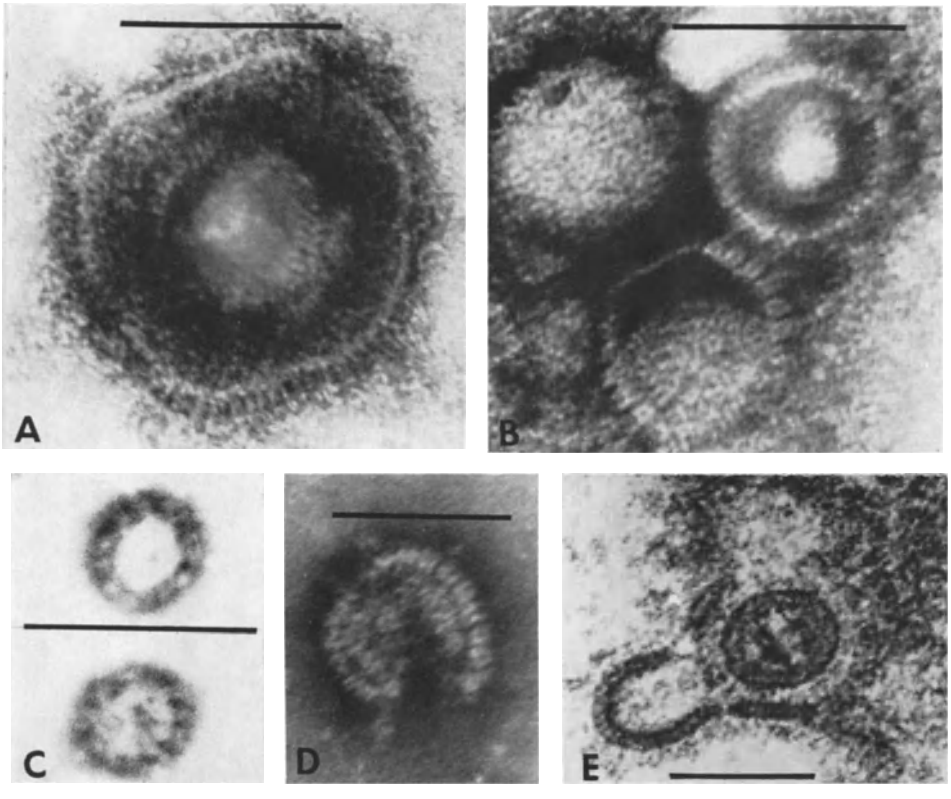

Fig. 1 A—E. Electron photomicrographs of herpes simplex virus. A enveloped nucleocapsid showing details of the internal structure of the envelope; B unenveloped nucleocapsids; C particles tentatively designated as consisting of a core and the inner capsid; D unenveloped nucleocapsid partially disaggregated in CsCl; E nucleocapsid in apposition to the inner lamella of the nuclear membrane; the photomicrograph shows details of a structure surrounding the nucleocapsid. Bar = 100 nm. A—D stained with silicotungstic acid (SPRING et al., 1968); E thin section of infected HEp-2 cell, SCHWARTZ and ROIZMAN, unpublished data

and moreover, the space between the membrane and particle is filled by a substance which seems to adhere to the particle rather than to the membrane (Fig. 1 E). This substance adhering to the nucleocapsid is not a property of all intranuclear nucleocapsids. This conclusion is based on the observation that the minimal distance between adjacent nucleocapsids in crystalline assays in the center of the nucleus is less than the minimal distance between adjacent nucleocapsids at the nuclear membrane. The second observation is by SPRING and ROIZMAN (1968) that infectious virus extracted from nuclei of infected cells differs from virus extracted from the cytoplasm with respect

to size and stability on centrifugation in solutions of high ionic strength. The infectivity of both nuclear and cytoplasmic virus however, is inactivated by phospholipase C with simultaneous release of choline. The treatment with lipase does not affect the structure of the nucleocapsid as seen with negative staining. It is perhaps of interest to report that TOPLIN and SCHIDLOVSKY (1966) reported that in thin sections and in negatively stained preparations the nucleocapsids from Burkitt lymphoma cells were "coated" with some amorphous material readily removed by proteolytic enzymes. It is most likely, however, that the material forming this coat is antibody made against the nucleocapsid (W. HENLE, personal communication).

To summarize, the existence of an outer envelope is firmly established. The existence of an inner envelope is less firm. The inner and outer envelope in thin sections account for the two outer shells — one electron opaque and one electron translucent. Some of the postulated properties of the inner envelope are that (i) it is electron translucent, (ii) it is readily permeable to phosphotungstic and silicotungstic acids, (iii) it contains lipid or lipoproteins which are unstable and tend to disperse in solutions of high ionic strength and, lastly, (iv) only nucleocapsids covered by an inner envelope acquire the affinity for the nuclear membrane and become fully enveloped.

4. The Outer and Middle Capsids

The most extensive studies of the outer capsid were those of WILDY et al. (1960). They reported that herpes simplex virus stained with phosphotungstic acid at low pH resulted in a better definition of the capsid structure at the expense of the envelope. Their data show that the surface of the nucleocapsid consists of 162 capsomeres arranged to form an icosahedron showing a 5:3:2 axial symmetry. The capsomeres on the surface of the nucleocapsid were described as prisms with hexagonal cross sections approximately 9.5×12.5 nm; since they fill partially with phosphotungstic acid it has been inferred that the distal end is hollow. The hole is estimated at 4.0 nm in diameter running down the middle along the long axis.

The evidence for the existence of another capsid (henceforth designated as the middle capsid) situated underneath the outer capsid comes from several sources. WILDY et al., (1960) described the existence of a "core" approximately 77.5 nm in diameter. According to their calculations a particle of this dimension could be obtained by stripping from the 105 nm nucleocapsid a shell of capsomeres 12.5 nm thick. The particle was seen only as a part of a disintegrating virion and never by itself. A particle of similar size was seen by SPRING et al. (1968) inher pes simplex virus partially disaggregated by centrifugation to equilibrium in CsCl (Fig. 1 D). The nucleocapsids seen in the centrifuged preparation ranged from a small minority lacking a few capsomeres of the outer capsid to numerous particles with only a few capsomeres still projecting from an inner structure impermeable to negative stain. In size and appearance the structure was similar to that seen by WILDY et al. (1960).

The size of the middle capsid may be deduced from published photomicrographs of negatively stained preparations and thin sections. The photomicrographs reported by STACKPOLE and MIZELL (1968) (Fig. 2G) and by TOPLIN and SHIDLOVSKY (1966) (Fig. 2A, B) show that phosphotungstic acid pene-

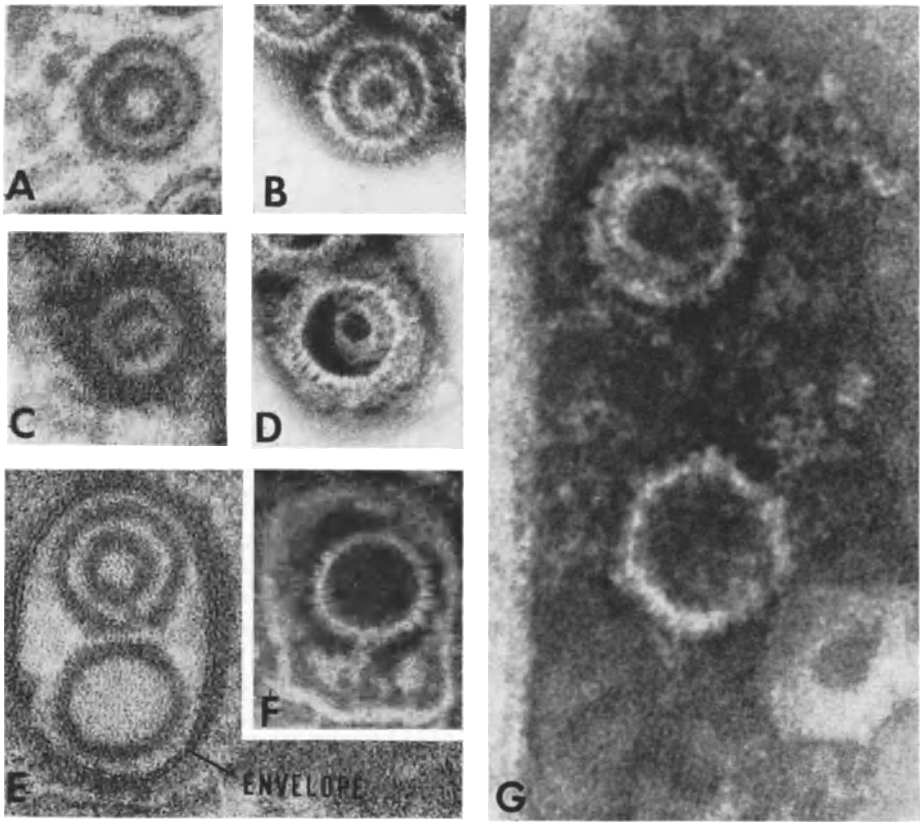

Fig. 2. Fine structure of herpesviruses isolated from Burkitt lymphoma (A—F) and from Lucké adenocarcinoma of the frog (G). A, B particles consisting of a core and a 3-layered capsid; C, D the same as A and B but coated by an amorphous material (inner envelope ?); E, F enveloped nucleocapsids; G naked nucleocapsids from Lucke adenocarcinoma. The upper nucleocapsid shows details of an internal structure. A, C and E thin sections, B, D, F and G negatively stained preparations. A—F, TOPLIN and SCHIDLOVSKY (1966). G, STACKPOLE and MIZELL (1968)

trates the inner structure of herpesvirus nucleocapsids extracted from frog adenocarcinoma and Burkitt lymphoma cells, respectively much more readily than that of the herpes simplex virion. The phosphotungstic acid delineates an inner shell which in the photomicrograph by STACKPOLE and MIZELL (Fig. 2G) has an outer diameter of 75—77 nm and an inner diameter of 45 nm. The outer dimension corresponds to the internal structure seen in partially disintegrated particles by WILDY et al. (1960) and SPRING et al. (1968). In thickness, and size, the shell corresponds to the concentric electron translucent shell seen in thin sections of nucleocapsids (Figs. 2A, B). The properties

and structural components of the internal capsid are uncertain. It does not have affinity for electron opaque salts of heavy metals and hence particles arrested in that stage of development could not be differentiated from particles consisting of cores and inner capsids.

5. The Core and Inner Capsid

EPSTEIN (1962a) and ZAMBERNARD and VATTER (1966) demonstrated by means of nuclease digestion the presence of DNA in the electron opaque or semi-opaque center core of the nucleocapsid of herpes simplex and of the virus found in adenocarcinoma of the frog, respectively. Evidence concerning the dimension of the core and the existence of an inner capsid surrounding the core have come from several different sources.

SPRING et al. (1968) described two small particles in preparations of herpes simplex stabilized with formaldehyde and centrifuged to equilibrium in solutions of CsCl. One particle banded between 1.37 and 1.45 gm/cm^3 and contained viral DNA. Electron photomicrographs of preparations stained with silicotungstic acid show a small particle approximately 25 nm in diameter; it was impervious to the negative stain and revealed no morphologic features. The particle was absent from unstabilized preparations centrifuged to equilibrium.

The second particle described by SPRING et al. (1968) banded in CsCl density gradients at a density of 1.325 gm/cm^3. Electron photomicrographs show a body 25 nm in diameter surrounded by bead-like projections which increased the diameter of the particle to about 45 nm (Fig. 1C). This particle was seen in fresh, unfixed lysates of infected cells. Particles of similar size were seen by others. Photomicrographs of negatively stained preparations of herpes type virus from Burkitt lymphoma cells by TOPLIN and SHIDLOVSKY (1966) show a centrally located body 25—30 nm surrounded by a shell 8 to 10 nm in thickness. Particles 30—40 nm according to SIEGERT and FALKE (1966) and 35—45 nm according to RABIN et al. (1968) were reported in the nuclei of cells infected with herpes simplex. The particles described by these authors consist of an electron opaque polygonal-shaped ring with a semi-electron translucent center. Similar particles somewhat larger in size (50 to 55 nm) and containing nucleoprotein were reported in preparations of bovine herpes virus by BOCCIARELLI et al. (1968). Particles of similar size were also reported by STACKPOLE and MIZELL (1968) in cell lysates and in nuclei of cells from adenocarcinoma of the frog and by EPSTEIN et al. (1968) in cells infected with the herpes virus present in fowl afflicted with Marek's disease. In thin sections of nuclei, the particle appeared as a semitranslucent body surrounded by an electron opaque shell. In negatively stained preparations the particle appeared impervious to PTA and poorly defined.

In light of the fact that the small 45—55 nm particles have been seen by a number of authors working with different herpesviruses and cells it seems rather unlikely that they are artifacts or adventitious contaminants. Most likely they are structural precursors of the virion. The association of the

25 nm particle with viral DNA (SPRING et al., 1968) suggests that it is the core. In size it corresponds to the semi-translucent center seen in the thin sections by SIEGERT and FALKE (1966), RABIN et al. (1968) and by TOPLIN and SCHIDLOVSKY (1966) (Fig. 2A) and delineated with phosphotungstic acid in photomicrographs (Fig. 2B) by TOPLIN and SHIDLOVSKY. The electron opaque shell surrounding the semi-translucent body in the photomicrographs reported by SIEGERT and FALKE (1966) and by RABIN et al. (1968) correspond in size to the bead-like projections around the 25 nm particle reported by SPRING et al. (1968). If the 25 nm particle is the core, it seems likely that the bead-like projections seen in negatively stained preparations and the inner electron opaque shell seen in thin sections represent the inner capsid.

6. Electron Microscopy of Herpesviruses — General Comments

The preceding analysis of the structure of herpesvirion was based on data obtained from thin sections and from negatively stained preparations. The data presented in the preceding section were highly selective; it seems desirable to comment on several findings and observations that did not fit the organization of the preceding sections.

(i) A study of the electron microscopic literature dealing with herpesviruses revealed a superstitious belief that the electron translucent shells seen in thin sections are artifacts due to shrinkage of the capsid and core during fixation. In consequence, no structure or function is assigned to the electron translucent shells. The fact that these shells are consistently seen in material fixed in many different ways seems to indicate that they are real. There is, moreover, abundant evidence from studies with negative staining techniques that the space corresponding to the electron translucent shell is not a void. Some artifacts however, may be unavoidable. Perhaps the most puzzling and disturbing observation is that the cores in nucleocapsids and virions differ with respect to their affinity for electron opaque heavy metal salts (MORGAN et al., 1959; FELLUGA, 1963; WATSON et al. 1964). The meaning of this rather frequent observation is obscure; it could reflect differences in permeability of the fixative or it could portend an unknown feature in virus assembly.

(ii) Almost without exception, herpesvirions in the cytoplasm of infected cells are sequestered within a space limited by a membrane. Occasionally, the membrane adheres closely to the virion and appears as an additional loose envelope. The membrane is probably a part of a tubule or duct in which virions accumulate in the cell (SCHWARTZ and ROIZMAN, 1969) and usually it is not seen in extracellular fluid. The exception is the herpesvirus seen in the extracellular space within the epithelium of renal tubules (LUNGER et al., 1965; ZAMBERNARD and MIZELL, 1965).

(iii) In evaluating the results obtained with negative staining two observations which detract from the value of the method should be noted. The first is that the process of staining and drying probably generates sufficient surface tension to cause considerable distortion of the material on the grid and may account for collapsed virions seen in preparations stored for a few

days prior to staining. "Collapsed" virions and nucleocapsids would be expected to have a slightly larger diameter than undistorted ones. The distortion introduced by the method may account for the variation in the size of the virion and nucleocapsid on record in the literature. The second point concerns variation in the penetrability of negative stains. Early studies suggested that nucleocapsids impervious to negative stain were "full" whereas those stained were devoid of cores. A recent paper by WATSON (1968) dispels this myth. Both phosphotungstic and silicotungstic acid are useful for analytical studies of the structure of the herpesvirion but probably not too useful for quantitative enumeration of "empty" or "coreless" particles.

B. Structural Components

1. Purification

Chemical analysis of viruses requires virus preparations of reasonable purity with respect to the specific component being analyzed. There have been numerous reports claiming preparations of virus free from certain host components. Thus consecutive differential (7000 g × 10 minutes and 20,000 g × 60 minutes), rate (30,000 g × 90 minutes through 15—50% w/w sucrose) and isopycnic (105,000 g × 40 hours in CsCl initial density 1.28) centrifugations preceded by chromatography on brushite columns (TAVERNE and WILDY, 1959) and followed by nuclease treatment will generally render virions free from detectable amounts of host nucleic acids at the cost of some 90% of the starting material (ROIZMAN and ROANE, 1964; SPRING and ROIZMAN, 1967; ROIZMAN, unpublished studies). However, none of the published procedures, too numerous to list here, satisfactorily separated an artificial mixture of unlabeled virus and radioactive debris from uninfected cells labeled with amino acids (ROIZMAN, unpublished studies).

2. Viral DNA

RUSSELL (1962) and BEN-PORAT and KAPLAN (1962) were, independently, the first to show by direct methods that herpesviruses contain DNA. To BEN-PORAT and KAPLAN must go the recognition for the demonstration that viral DNA differs significantly from host DNA with respect to its base composition — a finding of considerable usefullness in that it permits a simple and effective separation of the two. The base composition and size of the DNA of herpesviruses are shown in Tables 1 and 2, respectively. There is good agreement that while the DNA of all herpesviruses have a G + C content greater than that of the animal cells, the DNA of some herpesviruses have a much higher G + C content than that of others (BEN-PORAT and KAPLAN, 1962; DARLINGTON and RANDALL, 1963; RUSSELL and CRAWFORD, 1963; ROIZMAN et al., 1963; ROIZMAN and ROANE, 1964; KAPLAN and BEN-PORAT, 1964; RUSSELL and CRAWFORD, 1964; CRAWFORD and LEE, 1964; LANDO et al., 1965). There is also agreement that the DNA is double stranded (RUSSELL, 1962; DARLINGTON and RANDALL, 1963). There is less agreement on the

Table 1. *Base composition of herpes virus DNA*

Virus	Authors	Method	C + G mole %
Herpes simplex	BEN-PORAT and KAPLAN, 1962	Fractionation of ^{32}P labeled deoxyribonucleotides Spectrophotometrically (260 mμ)	74
Herpes simplex	RUSSELL and CRAWFORD, 1963	T_m	68
Herpes simplex	RUSSELL and CRAWFORD, 1963	Buoyant density in CsCl	68
Herpes simplex	RUSSELL and CRAWFORD, 1963	Spectrophotometrically (260/280 mμ at pH 3)	67
Herpes simplex	ROIZMAN et al., 1963	Buoyant density in CsCl	67
Herpes simplex	RUSSELL and CRAWFORD, 1964	Buoyant density in CsCl	68
Herpes simplex	LANDO et al., 1965	Fractionation of ^{32}P labeled deoxyribonucleotides	65 + 2.1
Herpes simplex, type 1	GOODHEART et al., 1968	Buoyant density in CsCl	68.3
Herpes simplex, type 1	GOODHEART et al., 1968	Buoyant density in CsCl	70.4
Pseudorabies	BEN-PORAT and KAPLAN, 1962	Fractionation of ^{32}P labeled deoxyribonucleotides	74
Pseudorabies	RUSSELL and CRAWFORD, 1964	Buoyant density in CsCl	74
Pseudorabies	KAPLAN and BEN-PORAT, 1964	Buoyant density in CsCl	73
Infectious bovine rhinotracheitis	RUSSELL and CRAWFORD, 1964	Buoyant density in CsCl	71
Equine herpes:			
(equine abortion)	DARLINGTON and RANDALL, 1963	Fractionation of deoxyribonucleotides	56
(equine abortion)	RUSSELL and CRAWFORD, 1964	Buoyant density in CsCl	55
(LK)	RUSSELL and CRAWFORD, 1964	Buoyant density in CsCl	56
Human cytomegalo virus	CRAWFORD and LEE, 1964	Buoyant density in CsCl	58
Bovine mammilitis virus	MARTIN et al., 1966	Buoyant density in CsCl	64
Equine abortion virus	SOEHNER et al., 1965	Buoyant density in CsCl and T_m	57

Table 2. *The molecular weight estimations of herpes simplex*

Virus	Authors	Method	Daltons
Herpes simplex	BEN-PORAT and KAPLAN, 1962	Average DNA content per virion	4.5×10^6
Herpes simplex	RUSSELL and CRAWFORD, 1964	Sedimentation coefficient	68×10^6
Herpes simplex	BECKER et al., 1968	Length and co-sedimentation with poxviruses in sucrose density gradient	100×10^6
Pseudorabies	KAPLAN and BEN-PORAT, 1964	Band width in CsCl	35×10^6
Pseudorabies	RUSSELL and CRAWFORD, 1964	Sedimentation coefficient	68×10^6
Infectious bovine rhinotracheitis	RUSSELL and CRAWFORD, 1964	Sedimentation coefficient	54×10^6
Equine herpes (LK)	RUSSELL and CRAWFORD, 1964	Sedimentation coefficient	84×10^6
Human cytomegalo virus	CRAWFORD and LEE, 1964	Band width in CsCl[a]	32×10^6
Bovine mammilitis virus	MARTIN et al., 1966	Band width in CsCl[a]	82×10^6
Equine abortion virus	SOEHNER et al., 1965	Sedimentation coefficient	$84—94 \times 10^6$

[a] The values obtained by this method are approximately one half the true value.

molecular weight but in all probability it ranges between 6 and 10×10^7 daltons (KAPLAN and BEN-PORAT, 1964; RUSSELL and CRAWFORD, 1964; CRAWFORD and LEE, 1964; BECKER et al., 1968). Unusual bases have not been reported. As indicated earlier (Section II, A, 5) the DNA is probably localized in the center (core) of the virion (EPSTEIN, 1962a; ZAMBERNARD and VATTER, 1966).

3. Viral Proteins

Acrylamide gel electrophoresis of herpes simplex virus solubilized in sodium dodecyl sulfate, urea, and β-mercaptoethanol revealed several proteins ranging in molecular weight to 120,000 daltons. The properties of the proteins are given in Section II, D, 2. The protein composition of nucleocapsids differs from that of virions (SPEAR and ROIZMAN, 1968).

4. Lipids

The presence of lipids is deduced primarily from the loss of biologic activity following exposure of the virus to lipid solvents (ROIZMAN and ROANE, 1963) and to lipases. Lipid is an essential constituent for the infectivity of both nucleocapsids extracted from nuclei and virions extracted from the cytoplasm of infected cells (SPRING and ROIZMAN, 1968). There have been few studies of lipid in infected cells (FALKE, 1967); the nature of the lipid is un-

known. It seems pertinent to mention here, however, that the source of genetic information for the synthesis of the lipid contained in the herpesvirion has not been unequivocably established. Virus grown in cells prelabeled with choline or in the presence of labeled choline became labeled and retained the label on isopycnic and rate centrifugations. Alas, virus was labeled not as well but equal tenacity, by artificially mixing unlabeled virus with labeled debris of uninfected cells (ROIZMAN, unpublished studies).

C. Physical Properties of the Herpesvirion and of the Structural Precursors

1. Buoyant Density of the Virion

Most of the available data concern herpes simplex virus. On isopycnic centrifugation in CsCl solutions the herpesvirion bands at a density ranging from 1.255 to 1.280 depending on four factors i.e.; (i) length of centrifugation, (ii) prior treatment of the cell lysate, (iii) virus strain and (iv) the cell in which the virus was produced.

In general, prolonged centrifugation in CsCl tends to increase the buoyant density. Thus the difference between the buoyant density obtained from preformed and self forming gradients centrifuged at the same rate for 5 and 48 hours respectively, may be as much as 0.02 gm/cm^3. Concomitant with the increase in buoyant density there is considerable disaggregation of virions. Herpes simplex virus may be stabilized by pretreatment with formaldehyde. The formalinized virus bands at a density of 0.015 gm/cm^3 higher than the untreated one (SPRING and ROIZMAN, 1967; SPRING et al., 1968).

Mutants of herpes simplex have been shown to differ in buoyant density under specified conditions of centrifugation (ROIZMAN and ROANE, 1961a; KOHLAGE, 1964; ROIZMAN and AURELIAN, 1965; SCHIEK, 1967; SCHIEK and SCHNEWEISS, 1968; EJERCITO et al., 1968). In addition, the buoyant density of the virus may change if it is grown in a different host (SPEAR and ROIZMAN, 1967). However, the density of any one mutant produced in any one host is constant and reproducible (ROIZMAN and AURELIAN, 1965; SPEAR and ROIZMAN, 1967). The modification of herpes simplex virus induced by the host is likely to be a reflection of the structural components of the envelope contributed by the host. The evidence that host components are present is deduced from the nature of the envelopment process and demonstrated more convincingly in agglutination tests with antibody against specific host membrane antigens (WATSON and WILDY, 1963). It is perhaps not too surprising that the difference between the buoyant density of various herpesvirus mutants may also be a reflection of the composition and structure of the envelope. The conclusion is based on observations that with few exceptions (SCHICK and SCHNEWEISS, 1968) mutants differing in buoyant density differ also with respect to surface characteristics such as elution from calcium phosphate gels, immunologic specificity, and with respect to their effect on the social behavior of infected cells (ROIZMAN, 1962a; ROIZMAN and ROANE, 1963; ROIZMAN and

AURELIAN, 1965; KOHLAGE, 1964; EJERCITO et al., 1968). The most plausible explanation for these covariant properties of the herpesvirus virion is that virus constituents of the envelope (i) confer immunologic specificity, (ii) determine the interaction with calcium phosphate gel and with CsCl ions and (iii) are responsible for the modification of host membranes underlying the alteration in social behavior of infected cells.

2. Buoyant Density of the Structural Intermediates

The buoyant density of the nucleocapsid stabilized by formaldehyde is 1.325 (SPRING et al., 1968). As mentioned in Section II, A, 5, a stabilized particle tentatively identified as the core banded in CsCl solution at a density of 1.37—1.45 gm/cm^3 whereas another particle tentatively identified as a core encased in an inner capsid bands at the same density as the nucleocapsid. Both particles appear to be unstable in CsCl solution.

3. Immunologic Specificity of Herpesvirions and of Structural Intermediates

WATSON and WILDY (1963) have shown in antibody agglutination tests that virions differ from nucleocapsids with respect to immunologic specificity. Recent neutralization tests with infectious herpes simplex virus extracted from nuclei and cytoplasm seem to support their conclusions (ROIZMAN, SPRING and SCHWATZ, 1969). The envelope of the virion contains host determinant antigens (WATSON and WILDY, 1963) in addition to viral antigens (ROIZMAN and SPRING, 1967). There is no information concerning the immunologic specificity of other structural components.

4. Degradation of the Herpesvirions and of Structural Intermediates

It has been reported that ethyl ether disrupts the envelope without affecting the nucleocapsid (WILDY et al., 1960). Phospholipase C removes nearly 95 % of labeled choline from virus, but it does not alter the hydrodynamic behavior of the virus (SPRING and ROIZMAN, 1968). The virion is unstable and tends to disaggregate on centrifugation in CsCl (SPRING et al., 1967). Detergents (Triton X, Nonidet P40, sodium dodecyl sulfate, sodium desoxycholate, etc.) also disaggregate the virus (SPRING, SPEAR and ROIZMAN, unpublished data). WILDY et al. (1960) reported that the nucleocapsid, in contrast to the virion disaggregates at pH 4, but remains stable following treatment with trypsin, ficin, papain, ethyl ether, and detergents. Studies on controlled degradation of herpesvirions are sorely lacking.

D. Architectural Components and Biologic Function

1. The Problems

The most important biologic function is the ability to reproduce on infection of a suitable host. This section therefore deals primarily with the features of the virion which determine the capacity to infect the cell. The

experimental modifications of the virion which render it noninfectious are dealth with in part here and in part in Section III, A, B. The covariation between certain physical properties of the virion and its effect on the social behavior of cells is discussed in Sections II, C, 1 and IV, B, 4.

2. Infectivity: The Role of the Envelope

Although both virions and nucleocapsids are present in infected cells, only virions are present in the extracellular fluids. The nucleocapsid is either not released or is unstable in the extracellular fluid (NII et al., 1968a). It would seem that the enveloped nucleocapsid is selected or best suited for the extracellular environment but the question nevertheless arises whether the nucleocapsid is capable of infecting cells. HOLMES and WATSON (1961), corroborated by SIEGERT and FALKE (1966) reported that enveloped particles more readily adsorbed to cells than naked ones, but they indicated that this may reflect only the size difference. WATSON et al. (1964) subsequently reported that in some preparations the number of plaque forming units exceeded the number of enveloped particles. This finding led to the conclusion that probably both kinds of particles were infectious albeit not necessarily with the same efficiency. This conclusion was challenged by SMITH (1964) who centrifuged herpes simplex virus to equilibrium in CsCl density gradients and found infectivity associated only with enveloped particles. *A priori* it would seem that, assuming CsCl is not deleterious separation of enveloped and nonenveloped nucleocapsids should be straightforward whereas considerable error is inherent in the particle counting technique. However, isopycnic centrifugation in CsCl is deleterious in that it causes disassembly of the herpesvirion (SPRING and ROIZMAN, 1967; SPRING et al., 1968). Moreover, electron microscopic comparison of the enveloped and nonenveloped forms recovered after centrifugation clearly indicates that naked nucleocapsids are more severely damaged by CsCl (SPRING et al., 1968). Since SMITH (1964) does not furnish the amounts of naked and enveloped particles at the beginning and end of the centrifugation, his results cannot be used to differentiate between the two conflicting hypotheses.

It seems clear from the foregoing discussion that the enveloped virus is infectious and that the answer to the question whether the nucleocapsid is infectious required unbiased separation of enveloped and unenveloped virus. In an attempt to overcome the problems inherent in the attempts to separate virions and nucleocapsids, SPRING and ROIZMAN (1968) compared the properties of infectious virus from nuclei and cytoplasm of infected cells. They found that infectious nuclear virus sediments more slowly in sucrose density gradients and is considerably less stable in salt solutions than cytoplasmic virus. However, the infectivity of both nuclear and cytoplasmic preparations was inactivated by lipases. More recent data (SPRING and ROIZMAN, unpublished data) indicate that nuclear and cytoplasmic virus differ also with respect to immunologic specificity in neutralization tests. The data are not sufficient to state categorically that an outer envelope is not required. It seems clear, however that (i) an intact outer envelope is not required and (ii) the integrity

of lipids associated with the virion or with the nucleocapsid is essential for infectivity.

3. Infectivity — Inactivation

In addition to detergents (SPRING and ROIZMAN 1968, BEDSON and GOSTLING, 1958) lipid solvents, and lipases, herpesviruses are inactivated by trypsin (GRESSER and ENDERS, 1961) alkaline and acid phosphatases (AMOS, 1953) nitrous acid (IVANICOVA et al., 1963), ionizing and nonionizing radiations (POWELL, 1959; ROANE and ROIZMAN, 1964a) and by heat (FARNHAM and NEWTON, 1959; KAPLAN, 1957; HOGGAN and ROIZMAN, 1959b; STEVENS and GROMAN, 1963; PLUMMER and LEWIS, 1965; SCOTT et al., 1961; PLUMMER et al. 1968; EJERCITO et al., 1968). The effects of all these agents are not in themselves unusual. However, heat inactivation in particular has been used to differentiate between different strains of virus. Because many inactivation studies cannot be done with purified virus in a menstruum free from compounds protecting or competing with the virus for the inactivating agent, conclusions that one virus is more or less stable than another may well be unfounded. The critique is particularly appropriate to attempts to rationalize differences in the kinetics of heat inactivation reported from different laboratories. One method to control inactivation rate is to work with an artificial mixture of two viruses in which one serves as a standard for comparative purposes (ROIZMAN and ROANE, 1963; ROIZMAN and AURELIAN, 1965; EJERCITO et al., 1968).

The stability of herpesviruses has been of considerable concern during the early studies of the kinetics of virus multiplication, perhaps because of their reputation as cantankerous viruses that are unstable and difficult to maintain. Herpesviruses are clearly not very stable in cell culture medium at 37°C or, in fact at any temperature above —70°C and this general finding has led to concerted efforts by some investigators to find means to stabilize it (WALLIS et al., 1962; WALLIS and MELNICK, 1965, 1968). In our experience herpes simplex is stable for years at —70°C and a 50% skim milk —50% cell extract in culture medium; the virus is also relatively stable for weeks at 4°C as a 10% cell extract in distilled water (MUNK and ACKERMANN, 1953; ROIZMAN, unpublished data). However, although the virus stored in distilled water retains its infectivity, it tends to become somewhat more unstable on centrifugation in sucrose and nonionic fluids than freshly prepared virus (SPEAR and ROIZMAN, unpublished data). Glycerol, sorbitol, sucrose, and solutions containing proteins other than skim milk and sera have been also used for stabilizing the virus (ZINSSER and TANG, 1929; HOLDEN, 1932; SPECK et al., 1951; WELLER and HANSHAW, 1962; BENYESH-MELNICK et al., 1966). WALLIS and MELNICK (1965) reported that 1.0 M Na_2SO_4 or 1.0 M Na_2HPO_4 stabilize the virus remarkably well against heat inactivation at 50°C. In our experience 1.0 M Na_2SO_4 is of no particular advantage at 4°C or at —70°C. Alas, not too many experiments can be done at 50°C in 1.0 M Na_2SO_4. In a more recent publication WALLIS and MELNICK (1968) showed that dimethyl sulfoxide protects herpesviruses from inactivation by freezing and thawing.

III. The Reproductive Cycle

> "Every experiment is like a weapon which should be used according to its specific function — as a spear is used to thrust, or a club to batter . . . Hence it is very important to discover the true active forces in experiments in order to know in what form they can best be applied".
>
> PARACELSUS in *Art of Medicine* 1/6, 456

A. Initiation of Infection

1. Adsorption

The rate of adsorption of herpesviruses to cells, like that of other viruses, is dependent on the volume of inoculum, the presence of cations, the metabolic state of cells but not, within 4°—37°C limits, on the temperature of incubation during absorption. Most of the available data concerning adsorption, penetration and uncoating were obtained from studies with herpes simplex virus. It takes approximately two hours to adsorb 200 p.f.u. of herpes simplex virus to 10^6 cells in 1 ml of fluid (ROIZMAN, 1962b, 1962c). The adsorption is even slower if the cells, normally adhering to glass during growth, are suspended prior to exposure to virus (ROIZMAN, unpublished data). Virions adsorb more readily than nucleocapsids (HOLMES and WATSON, 1961; SIEGERT and FALKE, 1966). The nature of the receptor is unknown. Cells naturally lacking receptors for herpes simplex viruses have not been described. However, HEp-2 cells exposed to parathyroid hormone (ROIZMAN, 1962b) lost temporarily the capacity to absorb virus. Thyroid hormone in the same test accelerated absorption (ROIZMAN, 1962c). Lack of receptors may explain the lack of susceptibility of normal human leukocytes for the herpesvirus extracted from Burkitt lymphoma.

2. Penetration and Uncoating

Adsorbed virus may be prevented from infecting the cell by the timely addition of antibody to the extracellular fluid. Adsorbed virus inaccessible to antibody is defined as having penetrated into the cell. Penetration is temperature dependent and requires expenditure of energy by the cell. HUANG and WAGNER (1964) found that once virus adsorbs to the cell, penetration, as defined, is relatively rapid. HOCHBERG and BECKER (1968) reported that after penetration into the cell, the virus becomes associated with hydrodynamically large structures. The virus was uncoated by existing cellular enzymes and naked DNA minus the capsid proteins was transported into the nucleus.

There have been numerous reports of electron microscopic studies of infected cells immediately after exposure of virus to cells. MORGAN et al. (1959), EPSTEIN et al. (1964a), HOLMES and WATSON (1961), SIEGERT and FALKE (1966) saw nucleocapsids free in cytoplasm and in "vacuoles" and concluded that virus is taken into the cells by pinocytotic vessicles in the manner of reovirus and vaccinia virus (DALES, 1962; DALES et al., 1965).

Subsequently, MORGAN et al. (1968) saw nucleocapsids free in the cytoplasm and in "vacuoles" and concluded that infection results from fusion of the viral envelope with the cell membrane leading to the release of the nucleocapsid into the cytoplasm in the manner of Newcastle diesase virus (MEISELMAN et al., 1967). MORGAN et al. (1968) list five steps in the initiation of infection i.e., (i) attachment, (ii) digestion of the viral envelope, (iii) digestion of the cell wall, (iv) passage of the nucleocapsid directly into the cytoplasm and (v) digestion of the capsid with the release of the core. The conclusion that the envelope is digested is based on electron photomicrographs showing distortion of the particles, and partial loss of the electron opacity of the envelope at the point of adhesion to the cell; MORGAN et al. (1968) suggest that the digestion is caused by an enzyme present in the virus. Similarly, the conclusion that the cell membrane is digested is based on electron photomicrographs showing the loss of electron opacity and the junction of cell and virus, and the authors this time suggest that the enzyme responsible for the digestion of the cell membrane is a component of the host.

The evaluation of the electron microscopic contribution to the studies of adsorption and penetration seems to call for several comments. Firstly, highly concentrated virus preparations must be employed for particles to be seen in thin section with any reasonable frequency. There is no knowledge at all as to how the cell reacts when confronted with large amounts of enveloped particles and whether infection at low multiplicities, the more usual mode of infection in nature, follows the events described at very high multiplicities of infection. Secondly, the microscopist's dilemma is frequently that of a viewer who tries to deduce from random stills the subject of a moving picture played by numerous actors most of whom have forgotten the script. In particular virus preparations used in electron microscopic studies are frequently cell lysates produced by sonication and containing viral debris, particles at different stages of maturation, as well as particles degraded during cell lysis. From past experience it may be estimated that the ratio of infectious units (plaque forming units) to virions and nucleocapsids is seldom greater than 0.01. With this in mind, the conclusions that virus particles in vessicles do not lead to productive infection of the cell or vice versa — all based on relative numbers of virions in vacuoles and in the cytoplasm — are not very meaningful.

3. Interference with Adsorption to Cells

Since 1962 a number of laboratories reported that sulfated polyanions, both natural (agar mucopolysaccharide, heparin) and synthetic, inhibit virus multiplication by preventing the virus from adsorbing to the cell (VAHERI and PENTTINEN, 1962; VAHERI and CANTELL, 1963; TAKEMOTO and FABISH, 1964; NAHMIAS and KIBRICK, 1964; TYTELL and NEUMAN, 1963; NAHMIAS et al., 1964; BENDA, 1966b; HADHAZY et al., 1966). Heparin was reported capable of stripping virus already adsorbed to the surface of the cell (HOCHBERG and BECKER, 1968). It has been reported that the effectiveness of the polyanion depended within limits on the degree of sulfation, and the size of molecule;

it was not dependent on the nature and degree of branching of the polysaccharide backbone (NAHMIAS et al. 1964). The effects of sulfate groups in agar mucopolysaccharides were neutralized by the addition of protamine (TYTELL and NEUMAN, 1963). The effects of other polyanions were abolished by dilution. Herpes simplex virus mutants unaffected by sulfate polyanions have been obtained (SOKOLOV et al., 1967).

B. Survey of the Reproductive Cycle

1. Information Content

One objective of the studies on the multiplication of herpesviruses is a complete description of the structure, functions, amount, and time of synthesis of all the products specified by the virus in the infected cells. At present the number of products and their functions are uncertain. According to current accounting practices, herpesvirus DNA carries information sufficient to specify the sequence of 133,000 amino acids. Herpesviruses are not as complex as some of the large DNA T phages and, moreover, the amount of information carried by the virus seems astronomical by comparison with the information content of papova and adenoviruses, which also multiply in the nucleus, or with that of myxoviruses and arboviruses, which also have an envelope. What then is the need for so much genetic information?

The answer is unknown. A point that is worth considering is that all of the genetic information carried by the virus may not be expressed in cell cultures. The argument is based on the fact that most laboratory strains were recovered at one time or another from sick individuals. Prior to infection of cells *in vitro* the information content of the virus was shaped and moulded for many millenia for better survival in the complex multicellular organism it normally infects. Unlike the small DNA and RNA viruses, herpesviruses have established in the course of evolution a unique relationship with the host they usually infect (ROIZMAN, 1965c). The main feature of this relationship is that following primary infection, and in spite of the appearance of antibody, herpesviruses survive asymptomatically in some specific tissue for the lifespan of the host. Perhaps even more extraordinary, there are reproducible subtle biochemical and biophysical differences among viruses isolated from recurrent infections occurring in different parts of the body (DOWDLE et al., 1967; EJERCITO et al., 1968; M. TERNI, personal communication); these observations suggest that the site of survival is determined by the virus. The capacity to coexist is not an indication that the virus is incapable of inflicting injury: herpesviruses frequently cause death or very severe illness in species other than their natural hosts. Thus herpes simplex virus infection of man is usually inapparent, infrequently serious, and rarely fatal; in the rabbit the virus causes severe damage to the central nervous system. Pseudorabies is a mild disease of pigs resembling herpes simplex in man; it is fatal in sheep and cattle. Virus B causes recurrent eruptions in old world monkeys reminiscent of recurrent herpes infections of man. As several unfortunate virologists have

involuntarily demonstrated, virus B infection of man is almost invariably fatal. In the light of these unique features of their natural history, it seems reasonable to postulate that herpesviruses express their genetic potentialities more fully and effectively in the hosts with which they coexist than in ones they destroy. Alas, it is difficult to carry out meaningful biochemical experiments in experimental animals infected with a virus native to them. The cell culture is best suited for this purpose, but it is not the native habitat of herpesviruses in the evolutionary sense. For this reason, it seems unlikely that the entire information content of herpesviruses will ever be determined from studies of the infection in cultures of dispersed undifferentiated cells.

2. Characteristics of the Cycle

Most of the available information concerns herpes simplex, pseudorabies, cytomegalovirus, equine abortion virus, and to a limited extent the virus observed in human (Burkitt) lymphoma cells. For the purpose of this discussion the reproductive cycle is best described in terms of the factors affecting four parameters i.e., (i) the duration of the eclipse terminated by the appearance of new virus, (ii) duration of the reproductive cycle, (iii) the yield of virus per cell (iv) virus release from cells.

(i) The duration of the eclipse varies from 3 to 8 hours for most herpesviruses. It is affected by the temperature of incubation (FARNHAM and NEWTON, 1959; HOGGAN and ROIZMAN, 1959b; SMITH, 1963), by the multiplicity of infection, and by prior infection of cells with another mutant (ROIZMAN, 1963a, 1965a). Once a minimum eclipse period is attained (3 hours for pseudorabies in rabbit kidney cells, 5 hours for herpes simplex in HEp-2 cells) it cannot be shortened by increasing the multiplicity of infection (KAPLAN and VATTER, 1959; ROIZMAN, 1963a).

(ii) The duration of the reproductive cycle of herpesvirus varies considerably. The cycles of herpes simplex in HEp-2 cells and that of equine abortion virus in LM cells last from 13 to 19 hours, depending on the multiplicity of infection and temperature of incubation; at 37°C and 50 plaque forming units per cell the cycle lasts 17 hours (ROIZMAN et al., 1963; O'CALLAGHAN et al., 1968b; KAPLAN and VATTER, 1959). The cycle of pseudorabies in rabbit kidney cells is somewhat shorter.

(iii) The virus yield from infected cells increases exponentially from the end of the eclipse phase until almost the end of the reproductive cycle. Under optimal conditions the yield of herpes simplex virus is 10,000 to 100,000 virions per HEp-2 cell. The best preparation of WATSON et al. (1964) contained about 10 virions per plaque forming unit; routine preparations of virus contain on the order of 100 to 1,000 virions per plaque forming unit (SMITH, 1963). Cells grown and maintained in monolayer cultures usually yield more virus than those suspended after infection (ROIZMAN and SPEAR, 1968). The relative amounts of enveloped and naked nucleocapsids in herpesvirus infected cells vary not only with the conditions of infection but also with the host species in which the virus is grown. High titer virus is more readily obtained in rapidly

growing cells maintained after infection in an enriched medium (ROIZMAN and SPEAR, 1968) at a pH (ROIZMAN, 1965b) and temperature (FARNHAM and NEWTON, 1959; HOGGAN and ROIZMAN, 1959b; SMITH, 1963; ROIZMAN, 1965a) optimal for that particular strain.

(iv) Infectious herpesvirions first appear inside the infected cells. The release is generally slow, and temperature dependent (HOGGAN and ROIZMAN, 1959b). Herpes simplex virus is released more readily from suspended cells and at 37°C than from those adhering to glass surfaces or those incubated at 34°C or below. Since virus growth is best at 34°C, the culture fluid may be discarded and the virus extracted by freezing and thawing, douncing, or sonicating the infected cells in a small volume of fluid. Freezing and thawing releases numerous aggregates from infected cells; brief sonication to disperse the virus has been recommended (SMITH, 1963). Some members of the herpes group are not very infectious once released into the extracellular fluid (COUNT et al., 1964; BRUNELL, 1967).

3. Nutritional Requirements

The nutritional requirements of herpesviruses do not appear to be unique. However, two interesting observations have been reported:

(i) HEp-2 cells infected with herpes simplex virus and maintained in Mixture 199 (MORGAN et al., 1950) produce more infectious virus than those maintained in EAGLE'S (1959) medium containing 1 to 4 times the recommended concentration of amino acids and vitamins. Calf serum is not required (ROIZMAN and SPEAR, 1968).

(ii) HENLE and HENLE (1968) reported that the production of viral products in Burkitt lymphoma cells is enhanced by arginine deprivation. The meaning of this observation is unclear: the multiplication of herpes simplex virus in cultures of human embryonic fibroblasts, chick embryo fibroblasts, and of monkey kidney cells is also not affected by absence of arginine (JENEY et al., 1967). However, herpes simplex virus does not multiply in the absence of arginine in cells in continuous cultivation (TANKERSLEY, 1964; ROIZMAN et al., 1965, 1967; BECKER et al., 1967; JENEY et al., 1967; INGLIS, 1968).

The differences between primary and continuous cell cultures with respect to the capacity to support virus multiplication in arginine free medium may reflect the size of the arginine pool (PIEZ and EAGLE, 1958; GONCZOL et al., 1967) or the presence of mycoplasma which degrade arginine (ROUSE et al., 1963). (Parenthetically, the failure to isolate mycoplasma on suitable media is not very reassuring since obligate intracellular forms may be present.) Arginine starvation of cells in continuous cultivation does not prevent adsorption, penetration, uncoating of herpes simplex virus (INGLIS, 1968), the reproductive events occurring during the first 4 hours after infection (ROIZMAN et al., 1967), and viral DNA synthesis (BECKER et al., 1967). There is general agreement that the effects of arginine starvation are more pronounced than the effects of deprivation of other amino acids essential for animal cells. One observation pertinent to the understanding of this phenomenon emerged

from recent analyses of viral proteins made in infected cells incubated in media with and without arginine (SPRING and ROIZMAN, manuscript in preparation). The data show that the probability that a viral protein is completed in arginine deprived cells is inversely proportional to the molecular weight and arginine content. In the absence of arginine five relatively large proteins rich in arginine are made in smaller amounts than arginine poor proteins of the same size.

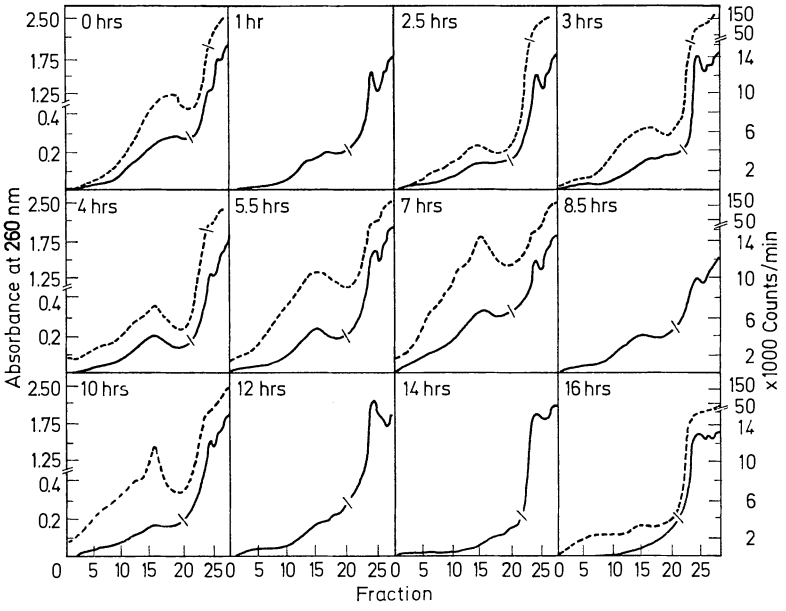

Fig. 3. The profile of polyribosomes extracted from HEp-2 cells infected with herpes simplex virus. The cells were pulse-labeled with ^{14}C reconstituted protein hydrolystae for 15 minutes immediately prior to extraction at times indicated on each profile. Solid line, absorbance; dashed line, radioactivity, SYDISKIS and ROIZMAN (1966)

4. The Patterns of Macromolecular Synthesis During the Reproductive Cycle

Information concerning DNA, RNA and protein synthesis is available for BHK-21, HEp-2, and KB cells infected with herpes simplex (ROIZMAN and ROANE, 1964; ROIZMAN et al., 1965; SYDISKIS and ROIZMAN, 1966, 1967; AURELIAN and ROIZMAN, 1965; SPEAR and ROIZMAN, 1968; HAY et al., 1966; FLANAGAN, 1967), for rabbit kidney cells infected with pseudorabies virus and for L—M cells infected with equine abortion virus (KAPLAN and BEN-PORAT, 1963, 1966a; HAMADA and KAPLAN, 1965; O'CALLAGHAN et al., 1968a, b). There have been no detailed studies of lipid or of carbohydrate synthesis in infected cells. The patterns for RNA, DNA and protein synthesis in HEp-2 cells infected with herpes simplex are shown in Figs. 3—5. The main features of the data are an initial period of decline in the rate of synthesis of all three macromolecules (0—3 hours after infection), a period of leveling off or increase in macromolecular synthesis (3—9 hours after infection) and lastly, a period of gradual and irreversible decline. These 3 periods coincide approximately

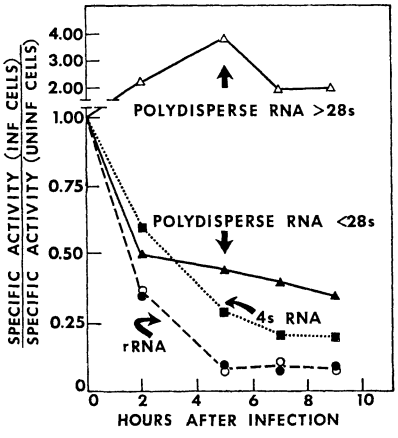

Fig. 4. The synthesis of various classes of cytoplasmic RNA in herpes virus infected HEp-2 cells. The cells were labeled with uridine for 30 minutes, then incubated for an additional 2 hours in medium containing unlabeled uridine. The RNA was extracted and electrophoresed in acrylamide gels. The relative specific radioactivity of the rRNA (28 S closed circles, 18 S open circles) and 4 S RNA (closed squares) were computed from the absorbance at 260 nm and radioactivity of RNA in the acrylamide gels and compared with the values for the RNA from uninfected control culture. The recovery of radioactivity of the polydisperse RNA was corrected for the recovery of rRNA in the corresponding cultures and also compared to values of the uninfected control (WAGNER and ROIZMAN, manuscript in preparation)

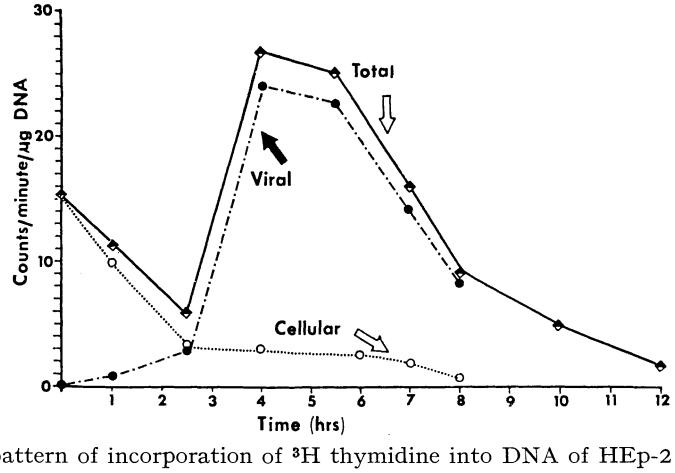

Fig. 5. The pattern of incorporation of ^{3}H thymidine into DNA of HEp-2 cells infected with herpes simplex virus. The cells were pulse-labeled for 15 minutes at different times after infection. The DNA extracted after the pulse was centrifuged to equilibrium in CsCl density gradients (SYDISKIS and ROIZMAN, unpublished data)

with (i) the inhibition of host macromolecular synthesis concomitant with the synthesis of nonstructural products specified by the virus (ii) the synthesis of components of the virus and (iii) assembly of the structural components into virions. The following sections deal with the synthesis of products specified by the virus and virus assembly. The inhibition of host macromolecular synthesis is discussed in Section IV, B, 2.

C. The Synthesis of Nonstructural Products Specified by the Virus

1. mRNA

By definition, viral RNA must be complementary to one of the strands of viral DNA. The operational definition of viral mRNA is that in addition to being complementary to viral DNA, it should function by specifying the structure of viral proteins. To date evidence has been presented that (i) transcription of viral DNA is required for virus multiplication (ROIZMAN, 1963b; SAUER et al., 1966; SAUER and MUNK, 1966; FLANAGAN, 1967), (ii) viral RNA is synthesized (HAY et al., 1966; FLANAGAN, 1967) and (iii) new species of polyribosomes appear in the cytoplasm of infected cells (SYDISKIS and ROIZMAN, 1966, 1967). The presence of viral RNA on the polyribosomes, although expected, has not yet been demonstrated.

In infected DK cells RNA annealable with herpes simplex virus DNA reached peak levels 6—7 hours after infection and declined slowly thereafter (FLANAGAN, 1967). The sedimentation coefficient of annealable RNA ranged between 12 and 32S but, whereas longer (>32S) molecules annealed with viral DNA, those less than 12S do not. From 6 to 14 hours after infection the sedimentation patterns and presumably the size of the RNA molecules annealable to viral DNA remained constant. The results of HAY et al. (1966) on the synthesis of herpes simplex virus RNA in BHK-21 cells agree with those of FLANAGAN on the general pattern of synthesis of viral RNA. They differ however in that HAY et al., ascribe to viral RNA a sedimentation coefficient of approximately 20S. Moreover, they indicate that a 4S RNA is also synthesized. The data of HAY et al., suffer from the fact that in crucial experiments BHK-21 cells were not uniformly infected as evidenced by the synthesis of ribosomal RNA and by the annealing of a substantial proportion of labeled RNA with cellular DNA.

It seems probable that the viral RNA described by FLANAGAN and by HAY et al. functions as the mRNA backbone of cytoplasmic polyribosomes, but this remains to be proven. There is evidence that the RNA is made in the nucleus (WAGNER and ROIZMAN, unpublished data). Nothing is known of (i) the source of the enzyme, (ii) the time of synthesis and the number of different kinds of viral RNA.

2. sRNA

In a series of papers published since 1965 SUBAK-SHARPE and co-workers (SUBAK-SHARPE and HAY, 1965; SUBAK-SHARPE et al., 1966; HAY et al., 1966, 1967; SUBAK-SHARPE, 1968) presented data in support of the hypothesis that herpesviruses synthesize at least one but probably several species of sRNA. The importance of these experiments stems from two unique functions of sRNA corresponding to two sites on the molecule. On one site the sRNA accepts a specific activated amino acid to form amino-acyl RNA. On the other site it contains bases complimentary to a codon on the mRNA. When this site and the codon are aligned on the ribosome the amino-acyl SRNA condenses with the terminal amino acid on the nascent peptide to form a

peptide bond. Parenthetically, virologists have long sought to understand the mechanisms by which viruses inhibit the synthesis of host proteins without affecting their own. It would perhaps be both instructive and amusing to speculate on the innumerable ways by which a virus could play havoc with host macromolecular synthesis were it but endowed with the information for the synthesis of its own sRNA and corresponding enzymes.

In the first paper SUBAK-SHARPE and HAY (1965) reported as evidence for the synthesis of new sRNA the coincidence of two activities in one fraction obtained by chromatography of RNA from infected cells on a methylated albumen-kieselguhr column. The fraction contained newly synthesized RNA complementary to viral DNA and RNA capable of accepting activated amino acids. The paper did not show whether the RNA complementary to viral DNA was the one accepting the amino acid. It is conceivable that small fragments of labeled viral mRNA could have eluted together with unlabeled host sRNA synthesized before infection. Two recent experiments are more convincing. In the first, SUBAK-SHARPE, SHEPHARD and HAY (1966) showed partial separation of arginyl-sRNA from infected and unifected cells. In the second they digested with ribonuclease T1 artificial mixtures of ^{3}H labeled amino-acyl-sRNA from uninfected cells and ^{14}C labeled amino-acyl-sRNA infected cells. The fragments were then chromatographed. Ribonuclease T1 hydrolizes RNA at guanine-phosphate bonds exclusively. If the hypothetical sRNA specified by the virus differs from the corresponding host sRNA with respect to the position of the first guanine base following the terminal triplet pCpCpA, it would be expected that after digestion with T1 at least one set of fragments attached to the amino-acyl group of the sRNA from the infected cell should differ from those of uninfected cells. The data do, in fact, show a difference between arginyl-acyl-oligonucleotides from infected and uninfected cells but here again it could be argued that the separation is due to differences in methylation patterns of RNA synthesized before and after infection.

Pertinent to this section are two observations. First, in HEp-2 cells infected with herpes simplex virus the synthesis of 4 S RNA and ribosomal RNA (as determined by electrophoresis in acrylamide gels) declines after infection. Ribosomal RNA synthesis however is inhibited more rapidly and more extensively than 4 S RNA (WAGNER and ROIZMAN, manuscript in preparation). Again however, it is not a all clear the 4 S fraction contains sRNA only. Second, in recent studies (MORRIS, WAGNER and ROIZMAN, manuscript in preparation) no differences have been found in the elution patterns of arginyl-sRNA extracted from infected and uninfected HEp-2 cells charged with corresponding enzymes, and chromatographed on reverse-phase freon-quaternary amine columns. The technique used in these experiments resolved two species of arginyl transfer RNA in both infected and uninfected cells. Among the many hypotheses that could account for this finding are (i) the virus does not direct the synthesis of a new transfer RNA in HEp-2 cells (ii) the cell and viral transfer RNA are indistinguishable and (iii) the chromatographic technique lacks sufficient resolving power.

3. Enzymes

Most of the data published in the past several years concern thymidine (TdR) kinase, thymidine monophosphate (TdR-MP) kinase, deoxyguanosine monophosphate (GdR-MP) kinase, deoxyadenosine monophosphate (AdR-MP) kinase, DNA nucleotidyltransferase (DNA polymerase) and deoxyribonuclease (DNAse) of cells infected with herpes simplex or with pseudorabies viruses. All of the enzymes studied to date concern themselves with DNA metabolism and few have been studied in detail. Although several enzymes are most probably synthesized according to information furnished by the virus the evidence on this point is based on precedents established with bacteria infected with T phages and is not unequivocal. The problem stems from the fact that the functions performed by these enzymes are common to both infected and uninfected cells. Acceptable evidence that an enzyme is "viral" would be (i) *in vitro* synthesis of the enzyme from a viral template; (ii) synthesis of the enzyme in an infected host rendered totally incapable of expressing its own genetic information and (iii) direct correlation of the amino acid sequence of the enzyme with coding sequences in the viral genome. The evidence available at present consists largely of physical or immunological differences between the enzymes recovered from uninfected and infected cells. In general it could be expected that viral and cellular enzymes performing the same function would differ with respect to immunologic specificity. Immunologic differences alone, however, are insufficient evidence that an enzyme in infected cells is "viral".

a) *DNA-Dependent RNA Polymerase.* The requirement for RNA synthesis early in infection is inferred from the observation that Actinomycin D inhibits the multiplication of herpes simplex virus (ROIZMAN, 1963b; SAUER et al., 1966; SAUER and MUNK, 1966). The source of the enzyme required for the synthesis of RNA is unknown. It is not clear whether the virus uses initially a host enzyme or whether it brings its own enzymes. In fact the possibility that the virus brings into the cell a small mRNA molecule has not been unequivocably excluded.

b) *TdR Kinase.* Thymidine kinase is a "scavenger" enzyme whose function is to convert TdR to TdR-MP. The enzyme is present in both "normal" and "neoplastic" cells grown *in vitro*. In BHK-21 cells activity increased as much as twenty fold from 2 to 8 hours after infection with herpes simplex virus (KLEMPERER et al., 1967). Thereafter, activity falls off.

KIT and DUBBS (1963a, b) showed in an elegant series of studies that TdR kinase activity was not essential for the growth of cells or of herpes simplex virus in cell cultures. First, they obtained a BUdR resistant strain of mouse fibroblasts lacking TdR kinase activity by growing the cells in media containing the analogue. TdR kinase activity was induced in these cells by herpes simplex and vaccinia viruses, but prevented by puromycin and actinomycin D. Subsequently they obtained TdR kinaseless mutants of herpes simplex virus by growing wild strains in BUdR resistant cells in the presence of the analogue.

MUNYON and KIT (1966) reported that in TdR kinaseless cells doubly infected with a wild strain of herpesvirus and a TdR-kinaseless mutant, the

activity of TdR kinase was considerably less than in cells infected with a wild strain alone. The significance of this finding is difficult to assess particularly since cells infected with a wild herpes simplex strain and a TdR-kinaseless mutant of vaccinia contained even less TdR kinase than the cells doubly infected with the wild and mutant strains of herpes simplex virus.

There are conflicting reports concerning the properties of the enzyme induced in infected cells. Compared with the enzyme from uninfected cells, the TdR kinase from infected BHK-21 cells was reported to have a low pH optimum and a low Km, and to be relatively stable at 40°C and insensitive to inhibition by deoxythymidine triphosphate (KLEMPERER et al., 1967). Moreover, the antigenic specificities of TdR kinase extracted from BHK-21 cells infected with herpes simplex and rabbit kidney cells infected with pseudorabies virus differed from those of uninfected cells (KLEMPERER et al., 1967; HAMADA et al., 1966). On the other hand TdR kinase activity of African green monkey kidney cells (BSC-1) could not be differentiated from that of cells infected with herpes simplex virus with respect to thermal stability and optimal temperature (PRUSOFF et al., 1965).

c) *TdR-MP, CdR-MP, AdR-MP and GdR-MP Kinases.* HAMADA et al. (1966) reported that TdR-MP kinase activity increased in rabbit kidney cells infected with pseudorabies virus whereas the activity of AdR-MP, GdR-MP and CdR-MP kinases remained unaltered. The same laboratory previously reported (NOHARA and KAPLAN, 1963) that TdR-MP kinase from pseudorabies infected rabbit kidney cells was more stable at 37°C than the corresponding enzyme from uninfected cells. PRUSOFF et al. (1965) observed a similar increase in TdR-MP kinase in African green monkey kidney cells infected with herpes simplex but, they were unable to differentiate between the properties of the enzyme in extracts of infected and uninfected cells. AdR-MP kinase from infected and uninfected cells could not be differentiated by antisera prepared against infected and uninfected cell extracts (HAMADA et al., 1966).

d) *DNA Polymerase (DNA Nucleotidyl Transferase).* A comprehensive discussion of DNA polymerases in herpesvirus infected cells was published by KEIR (1968). BRIEFLY, KEIR and GOLD (1963) reported that in BHK-21 cells infected with herpes simplex virus DNA polymerase activity of nuclei and of the mitochondria — microsome fraction increased two to six fold between 2 and 5.5 hours after infection. Actinomycin D prevents the increase in activity (KEIR, 1968). The polymerases extracted from infected and from uninfected cells differed with respect to immunologic specificity, heat stability (both with and without primer), primer requirements, and sensitivity to iodoacetamide and p-mercuribenzoate (KEIR, 1965; KEIR et al., 1966a, b; SHEDDEN et al., 1966). The enzyme assay developed by Keir takes advantage of the observation that the enzyme induced after infection is optimally active at 0.2 M NH_4^+ whereas the uninfected cell polymerase, endogenous DNAse and virus induced DNAse are all virtually inactive at that concentration of NH_4^+ (KEIR et al., 1966b; KEIR, 1968). Sephadex G-200 gel only slightly retards

DNA polymerase (KEIR, 1968). It is of interest to note that the DNA polymerase activity in bovine kidney cells infected with bovine rhinotracheitis virus is also more heat stable than the enzyme activity of uninfected cells (STEVENS and JACKSON, 1967).

It is of interest to note that HAMADA et al. (1966) could not differentiate in neutralization tests between DNA polymerase extracted from pseudorabies-infected and uninfected rabbit kidney cells. The results of HAMADA et al. are of interest: the rabbit is not a natural host for pseudorabies virus and this makes it even more improbable that virus and host polymerase are immunologically related. The enzyme neutralization tests were done with the serum of a rooster injected with sonicates of infected stationary (arrested by contact or density inhibition) cultures of rabbit kidney cells. The serum prepared against the uninfected extract lacked neutralizing activity. The effect of the serum made against the infected cell extract on the primer itself was not tested.

e) *Deoxyribonuclease.* An increase in DNAse activity (measurement at pH 7.3) in BHK-21-C13 cells infected with herpes simplex virus was reported by KEIR and GOLD (1963). The increase in enzyme activity leveled off 7 to 9 hours after infection (KEIR and GOLD, 1963; RUSSELL et al., 1964). More recently MCAUSLAN et al. (1965) and SAUER et al. (1966) reported an increase in activity of an "alkaline" DNAse in infected monkey kidney cells and L-cells, respectively. An increase in "acid" DNAse has been observed in herpes simplex virus infected HeLa and L cells (NEWTON, 1964) but not in monkey kidney cells (MCAUSLAN, 1965) or in KB cells (FLANAGAN, 1966).

The DNAse studied by KEIR and GOLD (1963) and subsequently by MORRISON and KEIR (1966, 1967) appears to differ from the host DNAse with respect to several properties. The induced DNAse is readily inactivated at 45°C, adsorbs to DEAE-cellulose, prefers Mg^{++} to Mn^{++}, and requires for optimal activity 50—60 mM Na^+ or K^+ whereas uninfected cell enzyme is stable at 45°C, does not adsorb to DEAE-cellulose, does not differentiate between Mg^{++} and Mn^{++} and is inhibited by Na^+ or K^+ at concentrations greater than 15 mM. Purified induced DNAse emerges in the void volume during gel filtration through Sephadex G-200 whereas uninfected cell enzyme is retarded. The enzymes extracted from BHK-21 and HEp-2 cells infected with herpes simplex virus or with pseudorabies are DNA exonucleases capable of degrading both native and denatured DNA to deoxynucleoside 5′ monophosphates whereas the enzyme extracted from uninfected cells is a DNA endonuclease effective primarily against denatured DNA. Rabbit antisera prepared against extracts of allotypic rabbit kidney cells infected with herpes simplex neutralized the DNA exonuclease from infected cells but not the endonuclease extracted from the uninfected cells (MORRISON and KEIR, 1967; KEIR, 1968).

f) *Other Enzymes.* FREARSON et al. (1965) reported that TdR-MP synthetase activity did not increase in mouse fibroblasts, HeLa, and rabbit kidney cells infected with herpes simplex virus.

An increase in CdR-MP diaminase in BHK-21 cells infected with herpes simplex virus has recently been reported by KEIR (1968). The enzyme catalyzes one of the reactions concerned with *de novo* synthesis of TdR-MP. The significance of the increase inactivity is unknown.

D. The Synthesis of Structural Components

1. Viral Proteins

This section was intended to deal with the time, intracellular site, and general patterns of synthesis of structural proteins of herpesviruses. However, the studies of the nature and number of herpesvirus structural proteins are still in their infancy and, at the moment there is no reliable method for differentiating between structural and nonstructural herpesvirus proteins in extracts of infected cells. For these reasons the scope of this section was expanded to a consideration of the total protein and viral protein synthesis in infected cells. The dual emphasis on protein and on antigen synthesis is prompted by the fact that immunologic techniques are frequently used to measure viral proteins. It is probably redundant to point out that while all proteins can be made antigenic, not all antigens are proteins.

a) *Characteristics of Protein Synthesis in Infected Cells.* As illustrated in Fig. 3, in HEp-2 cells infected with herpes simplex virus the rate of protein synthesis follows an initial period of decline (0—3 hours) a period of stimulation (3—8 hours) and, lastly, a period of gradual and irreversible decline (ROIZMAN et al., 1965; SYDISKIS and ROIZMAN, 1966). Analysis of proteins made at different times after infection revealed close agreement between the patterns of (i) specific activities of homogenates of pulse-labeled cells, (ii) the specific activities of peptides extracted from the cytoplasm of pulse-labeled cells (iii) the amounts of cytoplasmic polyribosomes recovered and (iv) the amounts of pulse-labeled nascent peptides bound to these polyribosomes at different times after infection (SYDISKIS and ROIZMAN, 1966). This led to the conclusion that (i) early and late inhibition and intervening stimulation of protein synthesis are due to the corresponding breakdown and reformation of polyribosomes, and (ii) the bulk of viral proteins is made on cytoplasmic polyribosomes.

The shift in the synthesis of proteins from cellular to viral is reflected in the profiles of polyribosomes (Fig. 6) in sucrose density gradients (SYDISKIS and ROIZMAN, 1966, 1967, 1968). The polyribosomes of uninfected HEp-2 cells are polydisperse, but with a peak fraction sedimenting at 170 S. These polyribosomes disappear between 1 and 2 hours after productive infection with herpes simplex virus (strain MPdk^-) and are replaced by polydisperse polyribosomes with a sedimentation coefficient of the peak fraction estimated at 270 S. The sedimentation coefficients of polyribosomes appearing in infected cells between 3 and 8 hours after infection in some instances reflect the nature of the infection. In DK cells abortively infected with MPdk^- virus, the peak polyribosome fraction has a sedimentation coefficient of 220 S. However, a mutant of MPdk^- designated MPdk^+sp which grows, but poorly, in both

HEp-2 and DK cells produces (Fig. 6) equal amounts of 220 and 270 S polyribosomes in the two cell lines (SYDISKIS and ROIZMAN, 1968). It is of interest to note that HEp-2 cells infected with MP*dk*⁻ produce predominantly enveloped nucleocapsids. DK cells infected with the same virus yields unenveloped nucleocapsids exclusively. The MP*dk*⁺*sp* virus produces in both cell lines relatively more nonenveloped than enveloped nucleocapsids (SPRING et al., 1968). Nevertheless in considering the role of cytoplasmic polyribosomes extracted 2.5 hours after infection and thereafter it should be pointed out

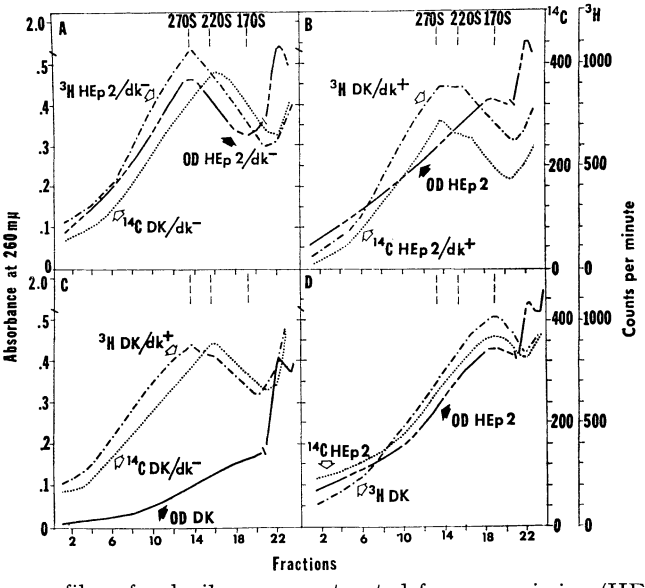

Fig. 6A—D. The profiles of polyribosomes extracted from permissive (HEp-2) and non-permissive (DK) cells infected with the MP*dk*⁻ and MP*dk*⁺*sp* mutants. Extracts of labeled cells were mixed and co-centrifuged with an extract of an excess of unlabeled cells. The absorbance monitored after centrifugation was due mostly to unlabeled extract. A Artificial mixture of extracts of HEp-2 cells infected with MP*dk*⁻ virus and labeled with ^{3}H amino acid, DK cells infected with the same virus and labeled with ^{14}C amino acids, and unlabeled HEp-2 cells infected with the same virus. B Artificial mixture of extracts of DK cells infected with MP*dk*⁺*sp* virus and labeled with ^{3}H amino acids, DK cells infected with MP*dk*⁻ virus and labeled with ^{14}C amino acids, and unlabeled uninfected DK cells. C Artificial mixture of extracts of DK cells infected with MP*dk*⁺*sp* virus and labeled with ^{3}H amino acids, HEp-2 cells infected with the same virus and labeled with ^{14}C amino acids, and unlabeled uninfected HEp-2 cells. D Artificial mixture of extracts of uninfected HEp-2 cells labeled with ^{3}H amino acids, DK cells labeled with ^{14}C amino acids and unlabeled HEp-2 cells (SYDISKIS and ROIZMAN, 1968)

that (i) viral mRNA has not been demonstrated on these polyribosomes (ii) nascent peptides on these polyribosomes have not been identified with respect to structure or immunologic specificity as being viral (largely because the bulk of the peptides become insoluble once they are released) and (iii) the correlation between the amount of 270 S polyribosomes and extent of envelopment will become significant if it is shown that viral envelope proteins are made on 270 S polyribosomes exclusively.

b) *Characteristics and Site of Synthesis of Viral Proteins.* Two recent reports deal with the proteins made in infected cells. FUJIWARA and KAPLAN (1967) reported that in pulse-labeled cells infected with pseudorabies virus, radioactive peptides first appeared in the cytoplasm; they were then chased into the nucleus. Nuclear labeled peptides reacted with antiviral antibody whereas cytoplasmic labeled peptides did not. SPEAR and ROIZMAN (1968) solubilized, then electrophoresed on acrylamide gels the proteins extracted from (i) cells pulse-labeled with amino acids at different times after infection, (ii) virus partially purified from cells labeled with amino acids after infection, and (iii) cytoplasm and nuclei from infected cells labeled with amino acids and then incubated in a medium containing unlabeled amino acids. The findings were as follows:

(i) A change in the pattern of protein synthesis was already apparent two hours after exposure of cells to virus. The polyacrylamide gel profiles remained more or less unchanged from 6 hours after infection. For descriptive purposes some 25 discrete radioactive protein bands which became apparent after infection were designated by a letter of the alphabet. By coelectrophoresis with poliovirus it was determined that the molecular weight of the proteins ranged to about 150,000 daltons. Assuming that the bands consisted of only one protein each, the proteins in the 25 bands accounted for some 20,000 amino acids i.e. for about 15 % of the probable information content of the virus (Section III, B, 1).

(ii) After infection labeled proteins appear first in the cytoplasm. The transfer of proteins from cytoplasm into the nucleus was slow and selective; less than half of the proteins synthesized during a short pulse appeared in the nucleus after a 3 hour chase. Moreover, proteins in 3 bands (a, n and p) appeared to be restricted to the cytoplasm.

(iii) The proteins labeled between 8 and 9 hours after infection form approximately 13 discrete bands (c, e, h, i, k, l, n, p, q, s, t, u, v) on electrophoresis. Partially purified enveloped virus labeled between 8 and 9 hours after infection yielded only 11 bands; the k and n bands were missing (Fig. 7). Comparison of a fraction consisting exclusively of enveloped virus with a fraction consisting of 80 % unenveloped nucleocapsids and 20 % enveloped nucleocapsids showed that the former contained relatively more I and p proteins. Band e appears to contain 3 proteins.

(iv) Preliminary surveys (SPEAR and ROIZMAN, unpublished data) revealed at least 2 proteins with affinity for lipids and 5 proteins rich in arginine and extractable with acid. STEVENS, KADO-BOLL and HAVEN (personal communication) also found five acid extractable proteins. All five proteins contain tryptophan and all but one are structural components of the virus.

c) *Viral Antigens.* Immunologic studies have concerned themselves with the time of synthesis, number (GOLD et al., 1963; RUSSELL et al., 1964; TOKUMARU, 1965a, b; HAMADA and KAPLAN, 1965; WATSON et al., 1966; FUJIWARA and KAPLAN, 1967), and site of accumulation of viral antigens in infected cells (LEBRUN, 1956a, b; ROSS and ORLANS, 1958; KAUFMAN, 1960;

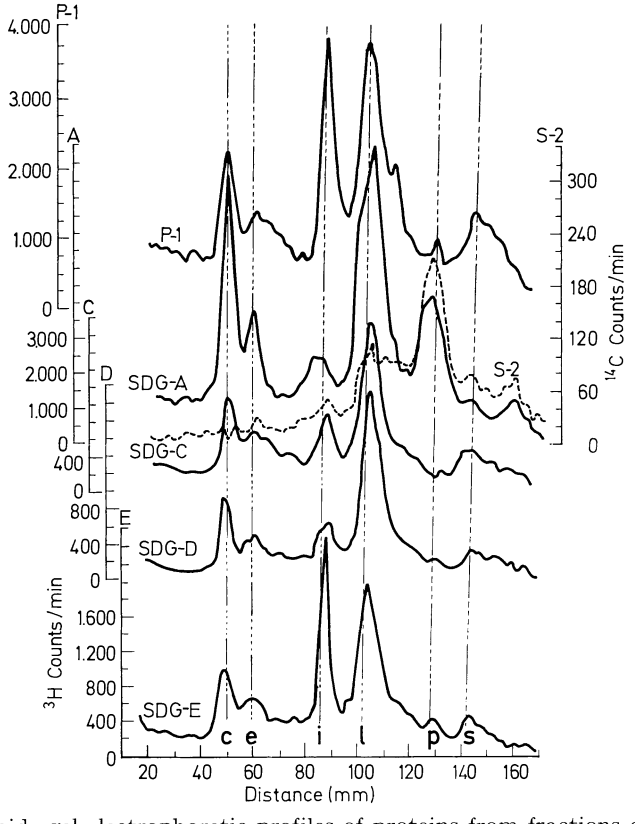

Fig. 7. Acrylamide gel electrophoretic profiles of proteins from fractions obtained in the course of partial purification of herpes simplex virus labeled from 6—24 hours after infection with ^{3}H-l-leucine. For purposes of alignment the fractions were co-electrophoresed with similar ^{14}C labeled fractions in appropriate combinations. For simplicity all but one ^{14}C profiles were omitted. P-1 is the pellet obtained after low speed centrifugation of cell lysate. S-2 is the supernatant fluid obtained after high speed centrifugation of cell lysate (sufficient to pellet virions and nucleocapsids). Sucrose density gradient fraction SDGE consisted entirely of enveloped nucleocapsids. SDGC and SDGE consisted of 80% unenveloped and 20% enveloped nucleocapsids. SDGA and SDGB (not shown) contained undifferentiated debris (SPEAR and ROIZMAN, 1968)

ROIZMAN, 1961; ALBRECHT et al., 1963; VOZZA and BALDUCCI, 1961; NII and KAMAHORA, 1963b; MUNK and FISCHER, 1965); O'DEA and DINEEN, 1957; BENDA, 1966a; RAPP et al., 1963; ROANE and ROIZMAN, 1966; ROIZMAN et al., 1967; SHIPKEY et al., 1967; GEDER et al., 1967a, b; 1968; GEDER and VACZI, 1968; ROSS et al., 1968; NII et al., 1968c). Before considering the results in detail it is necessary to discuss the source, preparation and testing of antibody. Briefly there are two sources of antibody i.e., (i) human or animal convalescent sera and (ii) hyperimmune sera prepared in a suitable animal. Each source of antisera has advantages and disadvantages. Convalescent sera are usually free from antibody reactive with uninfected cell extracts, but the antibody titers to viral antigens are not nearly as high as those obtained from properly immunized animals. Moreover, there may be considerable variation in anti-

body titer to the various viral antigens — a disadvantage in some respects which may nevertheless be very useful. Conversely, although hyperimmune sera can be made very potent and reactive with a large number of viral antigens, such sera must be absorbed repeatedly with an *excess* of uninfected cell homogenate to ensure absence of antibody reactive with host antigens. It should be noted in this connection that WATSON et al. (1966) reported an ingenious procedure for obtaining hyperimmune sera free of antibody reactive against cellular antigens. They immunized rabbits with extracts of infected rabbit cells grown in rabbit serum. The rabbits, the rabbit cells and the rabbit serum were all of the same allotype. The antisera were reported to be free from antibody directed against rabbit cells grown in cultures. Two examples of differences between convalescent and hyperimmune rabbit sera are pertinent here. First, in immunofluorescence tests several batches of human pooled γ globulin illuminated cytoplasmic viral antigens only; rabbit hyperimmune sera, on the other hand, contained antibody against viral antigens localized in both cytoplasm and nuclei of infected cells (ROIZMAN et al., 1967). Second, in the studies by TOKUMARU (1965 a, b) the most potent human convalescent serum gave 4 distinct precipitin lines in gel diffusion tests when tested against an infected cell extract. WATSON et al. (1966) using their rabbit hyperimmune serum obtained 12 precipitin lines in gel diffusion tests. However, the flattering comparisons should not be taken to mean that hyperimmune sera will invariably have antibody in equal titers against all of the antigenic products specified by the virus in infected cells or that they are invariably the reagents of choice.

A point should also be made concerning preparation of antibody reactive exclusively with specific antigens. Occasionally, procedures are found that yield antibody against specific classes of viral antigens. In this category falls the discovery that rabbit antibody made against boiled extracts of infected cells reacts exclusively with intranuclear viral antigens (ROANE and ROIZMAN, 1966; ROIZMAN et al., 1967). Most of the interest so far has been in the production of antibody to nonstructural proteins specified by the virus and conversely in the production of sera reacting with structural components of the virion only. Several comments concerning very obvious errors made in the past should be noted here. Firstly, the technique of immunizing rabbits with 3 hour infected cells in the expectation that such antisera will react with nonstructural components only or at least predominantly is probably not very useful for several reasons i.e., (i) not all of the virus adsorbed to the cell penetrates and becomes uncoated. The cell associated virus is exceedingly difficult to remove. Once the sonicated extract is injected into the animal there is every probability that the cell associated virus will infect the host. It is therefore most likely that the serum will contain antibody against virions and structural subunits. (ii) The fate of the coat protein of the infecting virus is unknown. It should be suspected until proven otherwise that at 3 hours after infection there is still enough coat protein in the cytoplasm of the infected cell to elicit antibody response. (iii) Structural components of the virus are

synthesized early in the reproductive cycle (SPEAR and ROIZMAN, 1968) in a manner analogous to those of vaccinia virus (SALZMAN and SEBRING, 1967). Parenthetically, absorption of such sera with intact virions does not make them more acceptable since intact virions will remove antibody to surface antigens only. For this reason lack of neutralizing antibody is not an indication that a serum is free from antibody to structural components of the virus. Secondly, the technique of injecting into animals *live* concentrated virus for the purpose of obtaining antibody reactive exclusively with structural components is also not very useful. Pseudorabies, herpes simplex, herpes B and other members of the herpesvirus group have a very broad host range and if the virus multiplies in the host, antibody against nonstructural viral proteins should also be expected. Even if infection aborts, as in the case of herpes simplex infection of DK cells (AURELIAN and ROIZMAN, 1965; SPRING et al., 1968), structural and nonstructural proteins may be specified by the virus.

One, and perhaps the unique, virtue of the preceding discussion is that it reduces considerably the number of reports contributing significantly to the problems concerning time and site of synthesis and the site of accumulation of herpesvirus antigens. Briefly, studies by RUSSELL et al. (1964) with a human convalescent serum and by SABIN (personal communication) with both hyperimmune rabbit sera and convalescent human sera show that the formation of complement fixing antigen in cells infected with herpes simplex virus takes place between 2 and 8 hours after infection. Thereafter, the complement fixing titer of the antigen remains unchanged.

The localization of herpesvirus antigens in infected cells has been studied with the aid of fluorescein and ferritin conjugated antibody; the 20 references cited at the beginning of this section are a relatively small sample of the total. Of the numerous studies published in the last few years, those on thin sections of infected cells stained with ferritin conjugated antibody (NII et al., 1968c) are the most recent and by far the most revealing. As might be expected the antigen was localized in the nucleus, the cytoplasm, and in the nuclear and cytoplasmic membranes. Antigen reactive with the conjugated antibody used by the authors was found in nearly every structure within the cell except in the nucleocapsid of the virus. Contradictory results have also been obtained with antibody conjugated with fluorescein in that viral antigen was found in nuclei alone, cytoplasm alone, and in both cytoplasm and nucleus. The earliest report is that of LEBRUN (1956a, b) who found that in HEp-2 cells infected with herpes simplex virus the antigen was first localized in the nucleus (24 hours after infection) and subsequently (60—72 hours) appeared in the cytoplasm. LEBRUN used a human serum. Other workers using human convalescent (ROIZMAN, 1961) and rabbit convalescent and hyperimmune sera (NII and KAMAHORA, 1963b) reported that viral antigen appeared first, between 1 and 6 hours after infection, in the cytoplasm at or near the nuclear membrane; nuclear fluorescence was not detected. Much later (ROIZMAN et al., 1967) it became apparent that the discrepancy between LEBRUN'S observation and those of subsequent investigators was due to differences in the specificity of

antibody used in these studies. Thus HEp-2 cells infected with herpes simplex virus were found to contain 5 immunofluorescent elements. Three (small nuclear granules, large nuclear granules, and an amorphous mass filling the nucleus) contained antigen which reacted with a rabbit serum prepared against boiled infected cell debris. A labeled pool of human antibody revealed antigens making up cytoplasmic granules and those responsible for the diffuse cytoplasmic fluorescence. All 5 immunofluorescent elements were demonstrable with a hyperimmune rabbit serum prepared against unheated infected cell debris. The antigens responsible for the diffuse cytoplasmic fluorescence and for the amorphous nuclear mass are synthesized early in infection and exist in a form which does not sediment on centrifugation at 79,000 × g for 2 hours. The antigens comprising the nuclear and cytoplasmic granules arise relatively late in infection and are readily sedimented on centrifugation at 79,000 × g for 2 hours. The identity of antigens responsible for the diffuse cytoplasmic fluorescence and amorphous nuclear mass are unknown. The cytoplasmic granule resembles in size accumulations of virions between the inner and outer layers of the nuclear membrane. Electron microscopy of nuclei shows several kinds of bodies and aggregations of subviral particles in the nucleus. The identity of the large and small granules is at the moment uncertain. Similar immunofluorescent elements were reported by Ross et al. (1968) and by Geder et al., (1967a, b, 1968) and by Geder and Vaczi (1968).

Since viral proteins are made in the cytoplasm and subsequently aggregates of these proteins form in the nucleus, it would be expected that at least some antigens would be found in both cytoplasm and nucleus. The most significant and also the most puzzling finding of the immunofluorescence studies cited above is that viral antigens were segregated in the nucleus or in the cytoplasm; within the limits of detection each antigen accumulated in one compartment only. One hypothesis that accounts for the various findings is that (i) some of the proteins made in the cytoplasm are not transferred into the nucleus in accord with the data of Spear and Roizman (1968) and that (ii) viral proteins made in the cytoplasm aggregate and acquire a new antigenic specificity on entering the nucleus. A necessary corollary to this hypothesis is that the milieu of the cytoplasm prevents viral proteins from aggregating before they reach the nucleus. *A priori*, in the light of the relatively high molecular weights of some structural proteins and a rate of transport into the nucleus which seems to be inversely proportional to size (Spear and Roizman, 1968) it would seem that aggregation of structural proteins in the cytoplasm would be most undesirable for the virus in that such aggregates would diffuse or be transported into the nucleus very slowly and inefficiently. Additional evidence to support or reject the hypothesis would have to come from studies with antibody against specific structural proteins of the virus.

2. Viral DNA

In the past several years information has become available on the synthesis of DNA of pseudorabies and herpes simplex viruses. The progress is due

largely to the fact that the DNAs of the two viruses have high G + C molar base ratios and are readily separated from cellular DNA by isopycnic centrifugation in CsCl density gradients (Table 1). It is perhaps relevant to note that very little is known concerning the synthesis of viral DNAs more closely approximating host DNA with respect to base ratios.

The most extensive studies were done on pseudorabies DNA synthesis in rabbit kidney cells (KAPLAN and BEN-PORAT, 1957, 1963, 1964, 1966a, b; KAPLAN, 1964; BEN-PORAT and KAPLAN, 1963). The system is particularly advantageous because only viral DNA is synthesized in rabbit kidney cells arrested prior to infection by contact (density) inhibition; host DNA synthesis along with cell division remain inhibited throughout the cycle (KAPLAN and BEN-PORAT, 1960). The studies dealing with the synthesis of herpes simplex DNA were done in HEp-2 cells (ROIZMAN and ROANE, 1964). Alas, optimal virus yields are obtained from young, rapidly growing cells (ROIZMAN and SPEAR, 1968). In HEp-2 cells infected during rapid growth period host DNA synthesis persists for several hours; for accurate determination of viral DNA it is necessary to centrifuge the extract containing DNA in CsCl solution (ROIZMAN and ROANE, 1964). O'CALLAGHAN et al. (1968a) studied the synthesis of equine abortion virus DNA in L-M cells. They separated viral and cellular DNA on methylated albumen-kieselguhr columns.

a) *Pattern of Synthesis of Viral DNA*. Two basic methods have been used to estimate the pattern of synthesis of viral DNA. The first involves determination of specific activity of viral DNA in cells pulse-labeled with radioactive thymidine at intervals after infection. The pattern obtained for herpes simplex virus DNA (SYDISKIS and ROIZMAN, unpublished studies) synthesis in HEp-2 cells is shown in Fig. 5. The salient features are that within a few hours after infection host DNA synthesis is almost completely replaced by viral DNA synthesis. The bulk of viral DNA is synthesized between 4 and 7 hours after infection; thereafter viral DNA synthesis declines slowly and irreversibly. On the basis of data shown in Fig. 5 it has been calculated that 6 to 9 hours elapse between the synthesis of DNA and formation of infectious virus.

The second method is based on a report by SALZMAN (1960) that fluorodeoxyuridine (FUdR) blocked the synthesis of vaccinia DNA. When FUdR was added to infected cultures during virus multiplication, DNA synthesis was arrested but virus assembly continued for several hours. SALZMAN concluded that infectious virus made in the presence of FUdR contained DNA made prior to the addition of the drug. Accordingly, a plot of virus obtained in the presence of the drug against the time of addition of the drug should yield the pattern of accumulation of viral DNA which becomes incorporated into virions. FUdR does not inhibit herpesvirus DNA synthesis in some established cell lines and therefore experiments similar to those reported by SALZMAN were done with iodouracil deoxyriboside (IUdR) (ROIZMAN et al., 1963) and bromouracil deoxyriboside (BUdR) (SIMINOFF, 1964). According to this method the DNA incorporated into virions is synthesized only two hours

earlier. The results obtained by the two methods differ, but they are not truly comparable. The direct method measures *all* viral DNA and makes only one critical assumption, i.e., that the thymidine pool inside the cell remains relatively constant throughout infection. However, only 10 to 15% of the *total* DNA measured by the first method becomes incorporated into virions and the pattern of total DNA synthesis may not be representative of the synthesis of DNA destined to become incorporated into virions (Section III, E, 1). The indirect technique purports to measure the DNA incorporated into virions only, but it is subject to two classes of errors with opposite net effects (ROIZMAN et al., 1963). First, the patterns obtained with these drugs may falsely indicate that DNA is made sooner than it is actually made. This could be if (i) penetration of the drug is delayed (ii) the phosphorylation of the drug is delayed or (iii) incorporation of small amounts of the drug into DNA does not affect the function of viral DNA. Second, the pattern obtained with the drugs may falsely indicate that DNA is made later than it is actually made. This could be if (i) the drug affects the ability of viral DNA to function in protein synthesis required for the formation of virions or (ii) the drug alters the ability of the cell to support virus maturation. Since the work was done the contribution of several class one errors was evaluated and found trivial (PRUSOFF et al., 1965). However, even though incorporation of IUdR into viral DNA could not be demonstrated in HEp-2 cells infected with herpes simplex virus (ROIZMAN et al., 1963), SMITH and DUKES (1964) reported the synthesis of defective virions, and KAPLAN and BEN-PORAT (1966b) were able to show that IUdR and BUdR become incorporated into DNA of pseudorabies virus and that the inhibition of virus assembly is due to the synthesis of fraudulent products. It may be concluded on that basis that the pattern of viral DNA accumulation obtained with halogenated pyrimidine nucleosides falsely indicated that the DNA is made later than it actually is. The magnitude of the error is not known. The question whether the pattern of synthesis of total viral DNA differs from the pattern of synthesis of DNA incorporated into virions is dealt with in Section III, E, 1.

b) *Requirements for Viral DNA Synthesis.* Two series of experiments have been reported showing that protein synthesis after infection is required to initiate the synthesis of viral DNA. In the first series, the exposure of herpes simplex virus infected HEp-2 cells to puromycin at any time between 0 and 3—4 hours after infection blocked the synthesis of viral DNA (ROIZMAN and ROANE, 1964). However, cells exposed to puromycin between 4 and 6 hours after infection i.e., after the onset of viral DNA synthesis, continued to incorporate radioactive thymidine into viral DNA albeit at a reduced rate. In view of the difference in size, base composition, and general structure of viral and cellular chromosomes, the observation that puromycin treatment immediately after infection blocks the onset of synthesis of viral DNA seems entirely reasonable. It indicates that viral DNA synthesis requires the participation of new enzymes made after infection. However, there is no simple, straightforward explanation for the observation that in cells treated with

puromycin after the onset of viral DNA synthesis, the rate of incorporation of thymidine into viral DNA is reduced. The greatest reduction in rate of incorporation of thymidine into viral DNA occurred within a short time after addition of puromycin and could reflect either a sudden change in the pool size of thymidine or a real change in the rate of synthesis of viral DNA. The first hypothetical explanation is probably trivial but it must be considered particularly in view of the possibility raised by NEWTON et al. (1962) that the size of the thymidine pool in the infected cells may change during the reproductive cycle. The second hypothesis if true, would indicate that puromycin interfered with the availability of a necessary rate-limiting constituent such as an enzyme or possibly the primer itself; it is perhaps pertinent to note that viral DNA extracted from puromycin treated cells behaved on isopycnic centrifugation in CsCl as if it were highly fragmented (ROIZMAN and ROANE, 1964). The objection to the second hypothesis is that the transfer of proteins from the cytoplasm into the nucleus is rather slow (SPEAR and ROIZMAN, 1968); and hence the absence of a hypothetical protein should not have become manifest immediately after addition of puromycin.

Similar results were obtained by KAPLAN and BEN-PORAT (1967) in rabbit kidney cells infected with pseudorabies virus. During the first hours after exposure to puromycin the rate of incorporation of thymidine into viral DNA dropped to one half that observed in untreated cells. Thereafter the rate decreased but not very appreciably. KAPLAN and BEN-PORAT concluded that changes in the rate of thymidine incorporation reflect changes in DNA synthesis and that pseudorabies virus DNA synthesis requires concomitant synthesis of one or more proteins. The authors speculate that the function of the short lived protein which must be synthesized continuously is to "prime" or initiate the synthesis of DNA. The conclusion is based on the observation that extracts from cells exposed to puromycin appear to contain all of the enzymes necessary to maintain DNA synthesis in a cell-free system consisting of cell extract, added deoxynucleoside triphosphates and heat denatured primer.

To sum up it seems clear that viral DNA synthesis requires *de novo* protein synthesis. Once initiated, viral DNA synthesis continues in the absence of concomitant protein synthesis but at a reduced rate. The reduction in rate, if true, is not readily interpretable for lack of information concerning (i) thymidine pool size before and after addition of puromycin (ii) integrity of replicating DNA in puromycin treated cells and (iii) secondary effects of puromycin on the intranuclear environment of the cell. There is, incidentally, no information concerning the stability *in situ* of the enzymes involved in the synthesis of viral DNA.

c) *Characteristics of Replication of Viral DNA.* KAPLAN and BEN-PORAT (1967) showed in studies reported between 1963 and 1967 that (i) pseudorabies virus DNA replicates in a semi-conservative fashion and (ii) less than one half of the DNA not integrated into virions and presumed available to function as templates is actually replicating.

d) *Site of Synthesis of Viral DNA*. Viral DNA synthesis takes place in the nucleus. For many years the conclusion was based on histochemical evidence (NEWTON and STOKER, 1958; MUNK and SAUER, 1964). Biochemical evidence was obtained in HEp-2 cells infected with herpes simplex virus. In cells fractionated immediately after a 5 minute pulse-labeling with ^{3}H thymidine, labeled DNA was found in the nuclear fraction (SYDISKIS and ROIZMAN, 1966). Autoradiographic data indicate that DNA synthesis is associated with an electron translucent matrix within the nucleus and not with any of the numerous electron opaque bodies present in the infected cell (SCHWARTZ and ROIZMAN, manuscript in preparation).

E. Virus Assembly

Information concerning the assembly of herpes viruses is derived from three sources i.e., (i) studies of the fate of viral DNA in infected cells (ii) the structure and characteristics of the herpesvirion detailed in part in Section II and (iii) electron microscopic studies of thin sections of infected cells.

1. Fate of Viral DNA

The withdrawal of viral DNA from the DNA pool into virions is slow and inefficient (KAPLAN and BEN-PORAT, 1966a; BEN-PORAT and KAPLAN, 1963). Viral DNA first becomes incorporated into a structure impermeable to DNAse but different from the virion (KAPLAN and BEN-PORAT, 1967). Biochemical and hydrodynamic characterizations of the structures containing DNA from the time DNA becomes DNase insensitive until the appearance of the complete virion have not been done. The question has arisen whether viral DNA made late in the reproductive cycle has a greater probability of becoming encapsidated. Ideally, random withdrawal would be expected if DNA and protein synthesis were completely and randomly dispersed throughout the cell. Some deviation from randomness would be expected if proteins and DNA are synthesized in separate compartments and must diffuse toward each other in order to aggregate; the DNA at the periphery of the compartment would have a greater probability of being encapsidated than the DNA near the center. The expected deviation from randomness, however, may be too small to be readily demonstrable in a system in which only 15—20 per cent of the DNA is incorporated into virions. BEN-PORAT and KAPLAN (1963) concluded that envelopment is random. In their experiments (i) a number of confluent rabbit kidney cultures were infected with 10 plaque forming units of pseudorabies virus per cell and incubated in a medium containing 25 μg of fluorouracil and 0.3 μg of thymidine per ml. (ii) At 3, 5, and 7 hours after infection the medium from various cultures was replaced with a medium containing ^{14}C thymidine. (iii) One hour afterwards the ^{14}C thymidine was diluted 1000 fold with ^{12}C thymidine. (iv) At intervals thereafter the medium was replaced with prewarmed fresh medium containing ^{12}C thymidine. The media removed at various intervals were assayed with respect to infectivity and with respect to the total amount of DNA contained in the virus released from the infected cells.

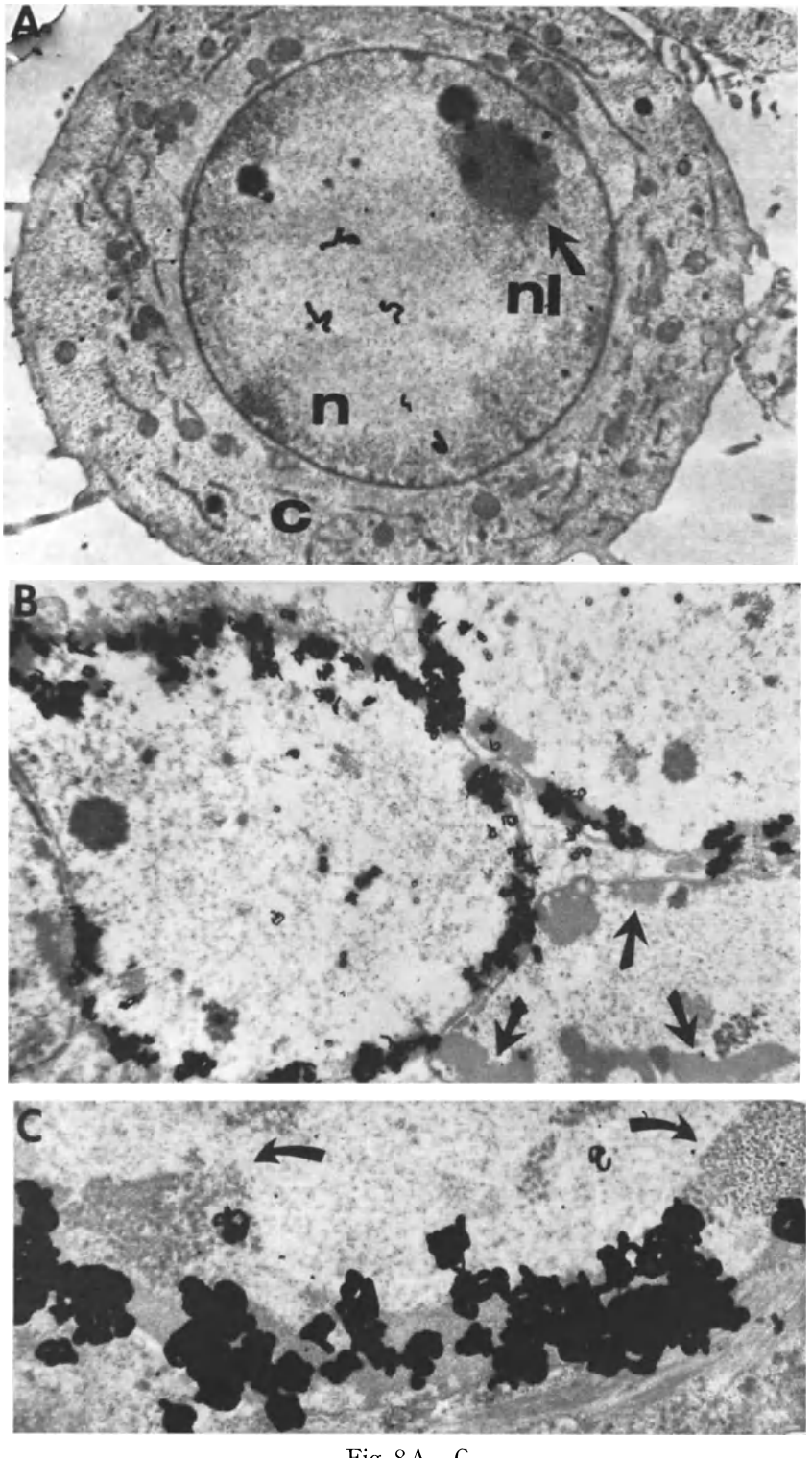

Fig. 8A – C

The findings were that, although the total amount of label incorporated in one hour beginning with 3, 5 and 7 hours after infection differed, the proportion of labeled DNA incorporated into virus was independent of the time of labeling. However, it seems appropriate to ask whether one or more variables in the experiments could have obscured a small deviation from randomness. First, the question arises whether fluorouracil could have minimized the effects of the difference in the time the cells were pulse-labeled. The question is pertinent particularly since it has been shown by REISSIG and KAPLAN (1962) that the drug causes the synthesis of nonfunctional proteins. Second, the question arises whether virus released from cells is a representative sample of the total virus made in the infected cell. The argument stems from the fact that only a small fraction of total virus is released.

2. Electron Microscopy of Encapsidation

Since 1954 over 100 papers have dealt with electron microscopy of cells infected with herpesviruses. Some of these papers have been cited in Section II, A. There is generally, very good agreement among investigators working with different herpesviruses and cells regarding the structures which emerge in the nucleus and cytoplasm of the cells following infection. There is less agreement regarding the significance and function of these structures. The major contribution of microscopy so far is to make apparent a picture of great complexity which must be accounted for. Some of the relevant observations may be summarized as follows:

(i) The nucleus of infected cells contains numerous relatively large bodies whose structure, origin and function are uncertain. Some of the bodies near the nuclear membrane are aggregated chromosomes (Fig. 8, B, C); this has been verified by autoradiography of cells labeled with thymidine-^{3}H prior to infection. Some bodies seem to originate from the nucleolus (Figs. 8A, 9A); at least bodies similar to those seen in infected cells have been reported in nuclei of uninfected cells injured by chemical inhibitors (BERNHARD, 1966). One or possibly two, bodies, however, are frequently associated with clusters of nucleocapsids and appear at present to be characteristic solely of herpesvirus infected cells (Figs. 8C, 9A—D).

(ii) The smallest particle seen in nuclei consists of an electron opaque ring surrounding a semi-translucent center (Fig. 9C). These particles are similar in

Fig. 8A—C. Electron photomicrographs of thin section autoradiography of HEp-2 cells infected with herpes simplex virus. A 4 hour-infected cell pulse-labeled for 15 minutes with thymidine-methyl ^{3}H prior to fixation. B Portions of 3 nuclei of 18 hour-infected cells labeled with thymidine-methyl ^{3}H prior to infection. Unlabeled thymidine was present in the medium during and after infection. Arrows point to aggregated chromosomes in a cell which did not synthesize DNA during labeling period. C Portion of one nucleus from section prepared as in B but photographed at higher magnification. Arrows point to two structures seen only in infected cells and which do not become labeled with thymidine during a short pulse. Abbreviations: *n* nucleus, *c* cytoplasm, *nl* nucleolus (SCHWARTZ and ROIZMAN, manuscript in preparation)

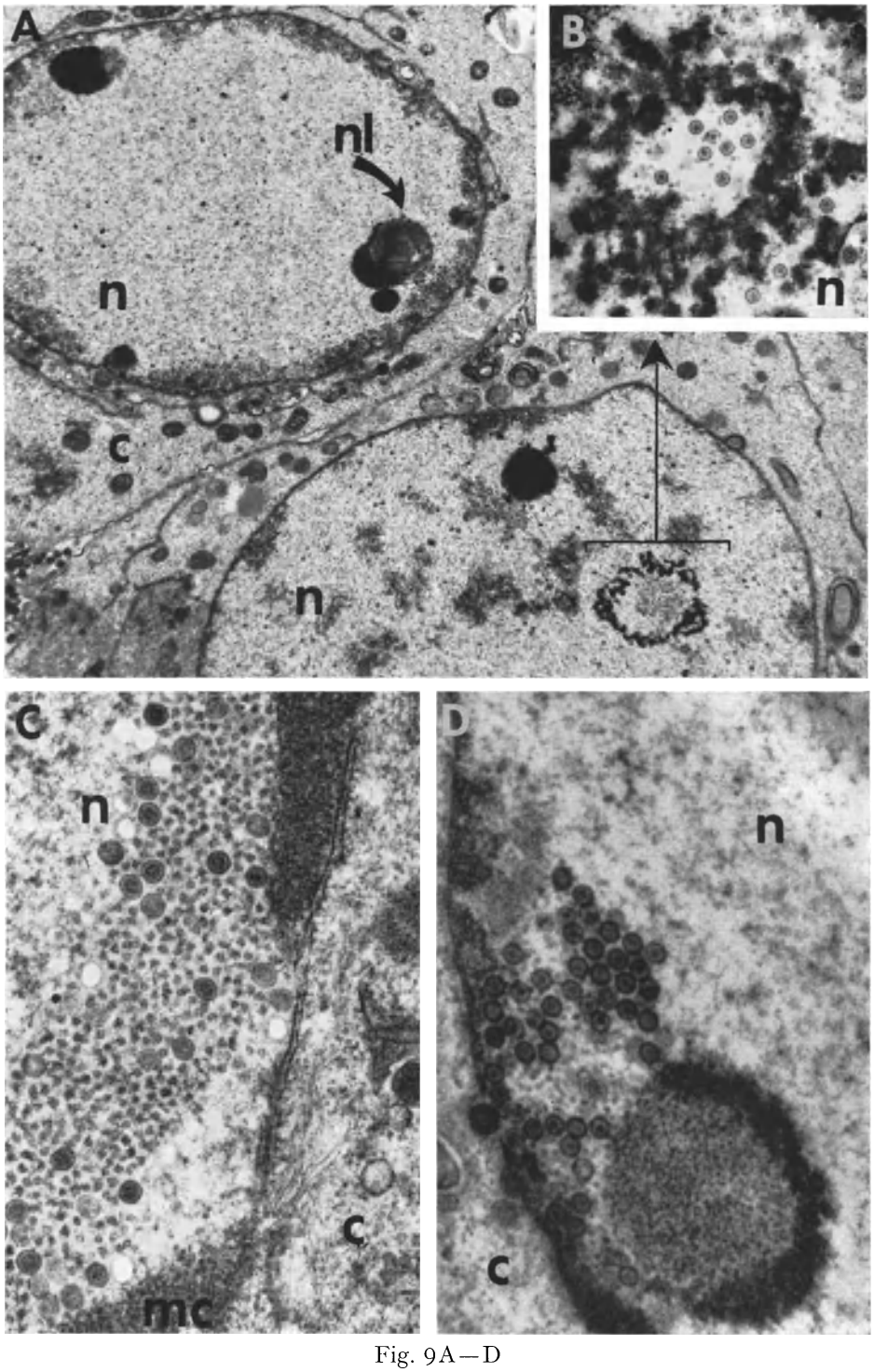

Fig. 9A—D

appearance to but smaller (30—40 nm) than the core and first electron opaque shell of the nucleocapsid (45 nm). The small particle is not adventitious virus since it has been seen in cells infected with herpes simplex (SIEGERT and FALKE, 1966; RABIN et al., 1968), with the virus associated with Marek's disease (EPSTEIN et al., 1968) and with Lucké adenocarcinoma (STACKPOLE and MIZELL, 1968). This particle could be an aberrant form of the core and inner capsid and as such it would be expected to accumulate or it could be a precursor. If the particle is a precursor, as postulated in Section II, its structure may be modified during subsequent assembly.

(iii) The distribution of nucleocapsids in the infected cell varies depending on the cell strain. In cells infected with most strains, unenveloped nucleocapsids are present solely in the nucleus. In cells infected with herpes simplex Type 2 (genital herpes), nucleocapsids are also present in the cytoplasm (Section III, F). There are indications that there are two types of nucleocapsids in nuclei. The first occurs in crystals (Fig. 10A) and in clusters with ring forms (Fig. 9) in or near some of the intranuclear bodies. Parenthetically, the nucleocapsids of all herpesviruses form intranuclear crystals but the frequency of crystal formation seems to vary from strain to strain. A characteristic of the nucleocapsids in crystals and clusters is that the outer electron opaque shells of adjacent nucleocapsids come very close together suggesting that the shells represent the outer covering of the nucleocapsids. The second type of nucleocapsid is present at the nuclear membrane. As shown in Fig. 1E and discussed in detail in Section II, A, 3 the nucleocapsid adhering to the nuclear membrane has an additional coat which in thin sections appear to be electron translucent.

It is not entirely clear from the electron microscopic observations what exactly is going on. One hypothesis that accounts for these findings but which is entirely without experimental basis is that nucleocapsids assemble very rapidly from modified ring forms. Once they are assembled, the nucleocapsids follow one of two pathways. They either acquire an internal envelope and then adhere to and become further enveloped by the nuclear membrane or they aggregate to form crystals. If this hypothesis is correct, a particle entering a crystal would not become enveloped and excessive crystal formation would suggest inability of the cell or of the virus to complete efficiently the maturation of the virus.

(iv) Certain inhibitors of macromolecular synthesis and analogues of ribose and deoxyriboside nucleosides (notably hydroxyurea, fluorouracil and iodoura-

Fig. 9A—D. Thin section of HEp-2 cells infected with herpes simplex virus. A Two cells containing several kinds of structures seen in infected cells. The nucleolus is partially disaggregated. B Enlargement of an intranuclear structure shown in A. Nucleocapsids frequently aggregate in the vicinity of this structure. C Aggregation of "ring forms" and nucleocapsids near the nuclear membrane. D A possible variant of the structure seen in B. Nucleocapsids frequently aggregate in the vicinity of this structure. Abbreviations: *n* nucleus, *nl* nucleolus, *mc* chromosomes aggregates near the nuclear membrane, *c* cytoplasm (SCHWARTZ and ROIZMAN, manuscript in preparation)

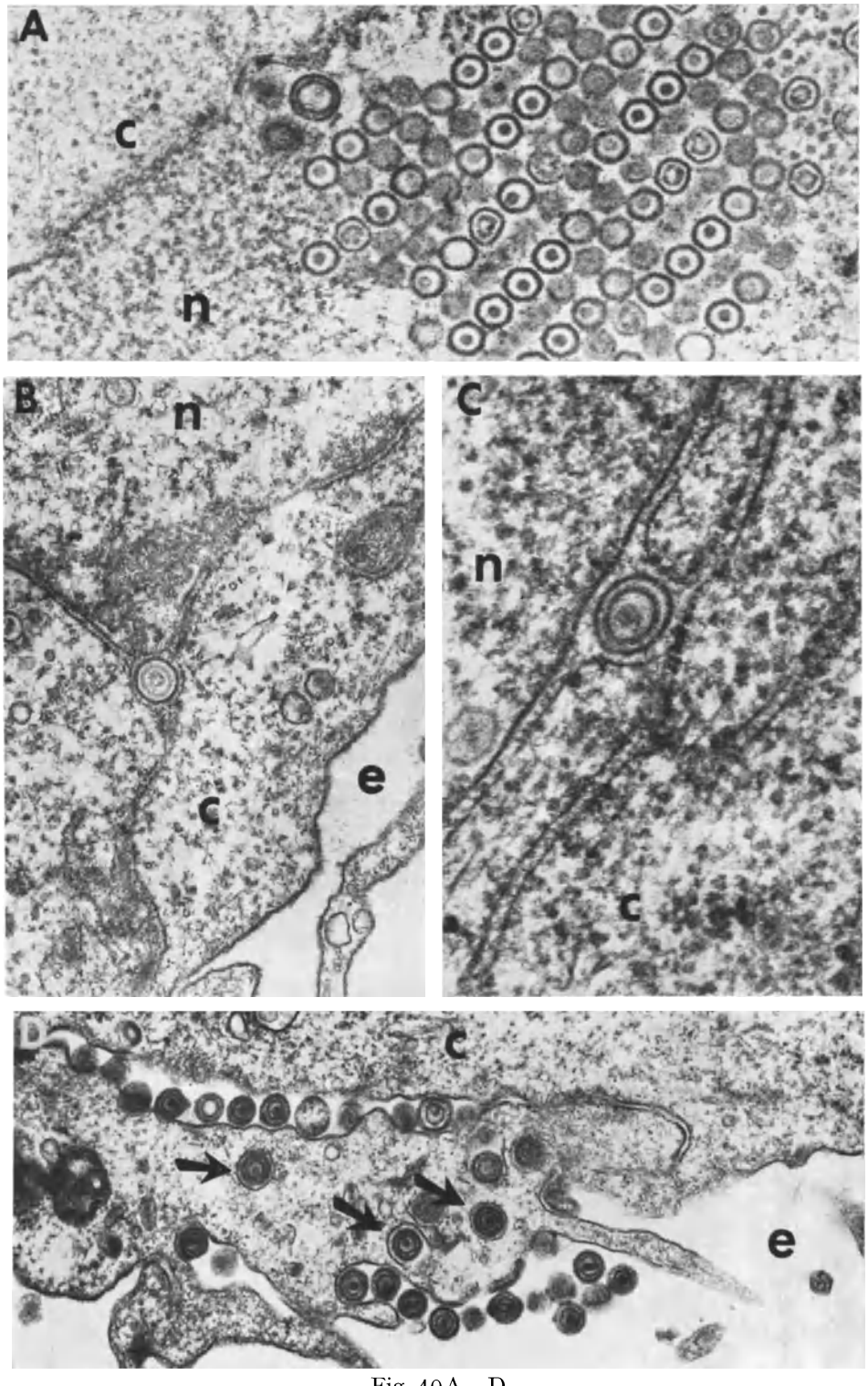

Fig. 10A—D

cil deoxyriboside) have been shown to cause an increase in the number of virions devoid of cores (Nii et al., 1968b; Reissig and Kaplan, 1962; Smith and Dukes, 1964). This raises two questions: first, what are the determinants of aggregation of capsid proteins and, second, how do the nucleosides affect these determinants. In view of the fact that the volume of the nucleus is far greater than the volume of all the nucleocapsids made in a single cell, it could be expected that if the aggregation of capsid proteins were haphazard, the number of coreless nucleocapsids would be far greater than the number of full ones. We may speculate that the DNA-protein core is assembled first and that the assembly of the capsid is initiated at some point on the core. With this hypothesis in mind the mode of action of the analogues is not entirely clear. It could be that the analogues cause the synthesis of fradulent proteins which aggregate spontaneously even in the absence of a core, or it could be that cores containing fraudulent DNA no longer fit into the capsid whose assembly they had initiated.

3. Envelopment of Nucleocapsids

There is generally good agreement that the nucleocapsid is enveloped by the inner lamellae of the nuclear membrane as it exits from the nucleus. The evidence consists of the following: (i) infectious nuclear virus is hydrodynamically smaller and less stable than cytoplasmic virus (Spring and Roizman, 1968) and (ii) electron photomicrographs show that naked and enveloped nucleocapsids are found in nuclei and cytoplasm, respectively, (Morgan et al., 1954, 1959; Stoker et al., 1958; Siegert and Falke, 1966) and that the nucleocapsid is most frequently "partially" enveloped only when it is in apposition to a thickened, modified portion of the inner lamellae of the nuclear membrane (Siegert and Falke, 1966; Shipkey et al., 1967; Darlington and Moss, 1968; Spring et al., 1968). There is less agreement on the question of whether the envelopment takes place uniquely at the nuclear membrane. On the basis of electron photomicrographs showing partially enveloped nucleocapsids it has been suggested that nucleocapsids may be enveloped by other membranes of the cell (Epstein, 1962b; Siminoff and Menefee, 1966). An excellent discussion on the difficulties inherent in the interpretation of the papers by Epstein and by Siminoff and Menefee is given by Nii et al. (1968a).

Fig. 10A—D. Thin sections of HEp-2 cells infected with herpes simplex virus. A Virus crystal. B Enveloped nucleocapsid between the lamellae of the nucleus at the junction with a tubule connecting nucleus with extracellular fluid. The nucleus contain an unenveloped nucleocapsid. C Enveloped nucleocapsid between the lamellae of the nuclear membrane at the junction with two tubules. Nucleus contains unenveloped nucleocapsid. D Enveloped nucleocapsid in the lumen of a tubule connecting with the cell surface. Enveloped nucleocapsids in sections cut perpendicular to the tubule (arrows) appear to be in "vacuoles". Abbreviations: *c* cytoplasm, *n* nucleus, *e* extracellular space. A 18 hours post infection; B, C 8 hours post infection; D 16 hours post infection; B, C, D (Schwartz and Roizman, 1969)

F. The Release of Virus from Infected Cells

The available data pertinent to virus release are that (i) infected cells do not lyse in the manner of bacteria infected with T phages (ROIZMAN, 1962a), (ii) virus release is temperature dependent (HOGGAN and ROIZMAN, 1959) and (iii) the herpesviruses released from infected cells consist predominantly of enveloped nucleocapsids (FELLUGA, 1963; NII et al., 1968a; SPRING et al., 1968). There is considerably less agreement on the mechanism by which enveloped nucleocapsids make their way from the nucleus to the extracellular fluid.

Electron photomicrographs show that, in the cytoplasm, the enveloped nucleocapsids are contained singly or in groups inside a structure bounded by a membrane. The last observation led MORGAN et al. (1959) to conclude that the enveloped virions are contained in a vacuole and that the virus is released by a process of "reverse phagocytosis". However, there is no data on the origin and content of the hypothetical vacuoles transporting the virus and moreover no evidence has even been presented that the "vacuoles" are closed structures. Recent studies (SCHWARTZ and ROIZMAN, 1969) revealed that the "vacuoles" of MORGAN et al. are in fact cross sections of tubules. A network of branched tubules 65 nm in diameter became apparent in HEp-2 cells 8 hours after infection with herpes simplex virus. The membranes limiting the tubules were continuous at one end with the outer lamellae of the nuclear membrane and at the other end with the cytoplasmic membrane. At 8 hours after infection enveloped nucleocapsids appeared at the junction of the tubules with the nucleus (Fig. 10B, C); nucleocapsids filled the tubules and appeared outside the cell 16 hours after infection (Fig. 10D). It is interesting that branched fibrils were also seen in infected cells with phase optics; they were stained with conjugated human convalescent antibody in immunofluorescence tests. The ducts as seen by the electron microscope and fibrils as seen by light microscopy were absent from uninfected cells and from DK cells abortively infected with the same virus.

Several comments concerning the structure and function of the tubules and the release of virus from cells seem appropriate.

(i) The tubules are not seen in uninfected HEp-2 cells. Nevertheless, the source of genetic information for the tubules is unknown. FELLUGA (1963) suggested that the structures bounded by membranes and countaining enveloped nucleocapsids are a host reaction to infection. Immunologic (ROANE and ROIZMAN, 1964; ROIZMAN and SPRING, 1967) light microscopic (ROIZMAN, 1962a) and electron microscopic (NII et al., 1968c) studies have established that nuclear and cytoplasmic membranes become altered after infection, presumably as a consequence of binding of products specified by the virus. It seems therefore reasonable to suggest that the tubules arose from a preexisting structure, possibly the reticuloendothelium of the cell modified by products specified by the virus.

(ii) There are no experimental data concerning the functions of the tubules. *A priori* it seems reasonable to conclude that assembled virus progeny should not accumulate in the same compartment as that in which the virus is un-

coated after entry into the cell. MORGAN et al. (1968) indicate that uncoating takes place directly in the cytoplasm. In our experience (ROIZMAN, 1963a, 1965a and unpublished studies) infected cells will uncoat a second superinfecting herpesvirus for at least 6 hours after infection with the first virus. It may be concluded that, for the first 6 hours after infection and probably longer, the cytoplasm is an unsuitable environment in that any virus accumulating in it will probably become uncoated. It is interesting therefore that, in HEp-2 cells infected with herpesvirus Type 2 in which intact tubules are infrequent, the cytoplasm is replete with unenveloped, partially disaggregated nucleocapsids (SCHWARTZ and ROIZMAN, unpublished data). The tubules form a third compartment in the infected cells; their function could be (a) to protect the enveloped nucleocapsids from being uncoated and (b) to provide pathways for virus release into the extracellular fluid. Precisely how the virus travels in the tubules is unclear; it could be that cell movement and contractions move the virus along the tubules.

(iii) It seems reasonable to expect that the method of egress suggested for herpesviruses might be a general property of all DNA viruses which multiply in the nucleus. It is interesting therefore that similar tubules were described in cells infected with SV_{40} (MAYOR et al., 1962; GRANDBOULAN, 1963; OSHIRO et al., 1967) and adenoviruses (DALES, 1962).

G. Regulation of the Reproductive Cycle

The keynote of life on Earth is reproducibility. Reproducibility inherently implies some forms of regulation. Life as we know it is the result of exceedingly rare errors compounded innumerable times — a true catastrophy in regulation. Viruses never cease to astonish us (considering the size and genetic potential) with the reproducibility of the growth cycle and with the fact that every one of the myriad progeny are an exact replica of the parent. This bespeaks a very high order of regulation; but the existence of groups with many mutants and strains differing from each other is mute evidence that this regulation, like that of their hosts, is not foolproof. The question before us is just which aspects of the reproductive cycle are regulated, how, and equally important, why. It is not profitable to consider the regulation of every event but some peculiar and unique problems associated with some groups and sequences of events should be noted. Before considering these, two points should be made. First, it seems desirable to stress that every succession of events, by virtue of the fact that it is a succession, is regulated. The second point is that in this discussion we should differentiate between two kinds of regulation. It seems self evident that some events are "committed" by a product of a viral gene whereas others, possibly of no consequence to the virus, occur simply because there is no viral product to interfere with the event.

1. The Synthesis of Nonstructural Components

Accustomed as we are to finding a rich and varied storehouse of transfer RNA, of enzymes involved in DNA synthesis, etc., in cultured animal cells,

it seems puzzling that DNA viruses carry such information and expend the host's energy to synthesize more molecules concerned with the same function. The question is particularly appropriate if one considers that, while the doubling time of most animal cells is nearly equal to the reproductive cycle of DNA viruses, the amount of cellular DNA and protein synthesized by the cell greatly exceeds the amount of viral DNA and protein made during the same period (KAPLAN and BEN-PORAT, 1960, 1966; SYDISKIS and ROIZMAN, 1966). Some explanations to this puzzle seem trivial as, for example, that the nonstructural components are evolutionary appendages dating back to a free living ancestor. Two other explanations seem far more probable. The first is that the cell grown in culture may bear little resemblance to its ancestors growing in a multicellular organism. Not all of the cells in the multicellular organism divide frequently and they would not, therefore, be expected to possess a full complement of enzymes required by the virus. Thus, one function of the nonstructural components would be to supplement the host; this is in accord with the observation that virus grows to higher titer in growing cells (SPEAR and ROIZMAN, 1968). Thymidine kinase is an example of a nonstructural component which is clearly in this category. The second hypothetical explanation is that some of the nonstructural products specified by the virus are endowed with specificity either with respect to the host, or to the virus. As considered in more detail in Section IV, B, 2, one obvious prerequisite of the inhibitors of macromolecular synthesis induced by viruses is that they differentiate between viral and cellular structures performing a similar function. Because of differences in size and structure of genetic material it seems probable that, in the environment of the cell, viral polymerase may be uniquely suited to duplicate viral DNA just as host enzymes may be needed to duplicate host genetic material. Parenthetically, if this were true, measurements of the enzyme in tests characterized by a lack of specificity have a limited value.

Assuming that the function of nonstructural products is in part to supplement host functions and in part to differentiate between host and viral functions, the question arises as to why so few of the functions of the host are supplemented. It seems puzzling, for example, that viral enzymes synthesized in the host are usually those concerned with thymidine utilization and not, for example, those concerned with the deoxypurine utilization. Experimental data bearing on this question are totally lacking. It could be that thymidine and its derivatives have a regulatory function in uninfected animal cells and their availability in the nondividing cell may therefore be controlled.

2. Regulation of the Synthesis of Structural and Nonstructural Components

The most persuasive argument for considering this topic is that, if viral DNA contains only one, nonrepeating codeword for each of the proteins specified by the virus, and if the DNA is transcribed *in toto* without interruptions, there would be as many enzyme molecules of each kind as there were structural subunits. For each herpesvirus containing 162N subunits (where N equals the number of identical subunits per capsomere) there would be

162N molecules of each enzyme. This enormous excess of enzymes is not found. Another proposal is that the DNA contains only one, nonrepeating, codeword for each enzyme and 162N repetitions of the codeword for the structural protein; however the difficulty now is that far more codewords would be required than there is space on viral DNA. Elimination of the two extreme hypotheses leaves only one alternative: regulation of the amount and time of synthesis. Phage work has led to the suggestion that viral proteins are synthesized sequentially: parental DNA serves as template for non-structural and a few structural proteins synthesized early in infection whereas progeny DNA serves as template for the bulk of the structural protein. This hypothesis raises questions concerning (i) how the synthesis of the late proteins is contained early in infection (if at all) and (ii) how the synthesis of "early" proteins is restricted late in infection.

Several interesting and very important experiments attempting to deal with the second of the two problems have been reported (KAMIYA et al., 1964, 1965; ZEMLA, 1967) and merit discussion. In the first of the papers, KAMIYA et al. (1964) reported an *in vitro* system measuring collectively the enzymes involved in the incorporation of deoxynucleotides into DNA. The authors showed that the limiting enzyme increased in activity during the first 6 hours, leveled off between 6 and 10 hours, and subsequently decreased. However, no leveling off or decrease in enzyme activity was observed in extracts of infected cells grown in a medium containing BUdR .This led to the conclusion that (i) the enzymes are regulated (ii) substitution of BUdR for thymidine interferes with the regulation and (iii) regulation was dependent not on presence of DNA *per se* but upon the presence of newly synthesized competent DNA. The questions thus arise as to what accounts for (i) the leveling off of enzyme activity between 6 and 10 hours after infection and (ii) the decrease in activity of the enzymes thereafter. Of the subsequent papers, one by KAMIYA et al. (1965) and one by ZEMLA et al. (1967) deal with these questions and conclude that between 6 and 10 hours after infection, enzyme synthesis is arrested whereas after 10 hours the decrease in enzyme activity is due to leakage of intracellular protein into the extracellular fluid as a consequence of viral release.

The loss of enzyme into extracellular fluid appears to be "regulation" by omission. It could be argued that if retention of enzymes were a selective advantage, mutants would have been found which did not cause enzyme leakage. In the light of the finding that at least 80% of total viral DNA is not encapsidated, it does not seem likely that retention of enzymes would be advantageous. In a different category is the conclusion that between 7 and 10 hours after infection enzyme synthesis is inhibited. This implies that regulation is "committed" by a product of a viral gene. For this reason, the evidence is worth examining in some detail.

Briefly, KAMIYA et al. (1965) showed that significant leakage of protein begins 5—8 hours after infection. However, the protein responsible for the leakage is synthesized beginning approximately 4 hours after infection. This

is shown by measuring loss of enzymes and leakage of proteins from cells exposed to puromycin at different times after infection. Thus there was no leakage at 15 hours from cells treated with puromycin beginning at 4 hours of infection. Cells treated at 7 hours or later leaked as badly as untreated cells. Between 4 and 7 hours loss of enzyme and total protein was dependent on the time of exposure to the drug. With this experimental background the authors asked themselves whether the leveling off of enzyme activity between 6 and 10 hours was due to (i) inhibition of enzyme synthesis or (ii) concomitant synthesis and loss of enzyme. To answer this question two sets of cultures were infected and incubated in medium free of serum. At 7 hours after infection one set received puromycin. The treated and untreated cells were then assayed for loss of enzyme activity from cells and leakage of proteins into the extracellular fluid. The argument was that if there is concomitant synthesis and leakage of enzyme, it would be expected that the enzyme loss should be greater in cultures treated with puromycin. The results show that treated and untreated cultures cannot be differentiated with respect to (i) the rate of enzyme loss and (ii) the rate of protein leakage. However, the interpretation of this experiment hinges on whether, at 7 hours after infection there was any protein synthesis for puromycin to inhibit. The basis for this question is two-fold. First, 7 hours in pseudorabies infected cells correspond roughly to 9 hours in herpes simplex virus infected cells. By 9 hours of infection 90% of the proteins synthesized during infection have already been made (SYDISKIS and ROIZMAN, 1966, 1967). Second, the authors' argument is not supported by the evidence they present. Thus if late proteins were synthesized at 7 hours and if puromycin were to inhibit the synthesis of late proteins, it would be expected that protein leakage from puromycin treated and untreated cells would differ. As indicated above, the leakage of protein from treated and untreated cells was exactly the same. It must be concluded therefore that no evidence emerged from this experiment that there was protein synthesis for the puromycin to inhibit between 7 and 10 hours after infection.

It is perhaps interesting to note that in another paper, KAPLAN et al. (1967) again reexamined the synthesis of enzymes in infected cells treated with inhibitors. In this study the authors compared the synthesis of TdR-MP kinases and TdR kinase in cells treated with actinomycin D 4 hours after infection with pseudorabies virus. They found that in infected untreated cells (controls) both enzymes increased in activity until about 6 hours after infection, then leveled off. In infected treated cells TdR-MP kinase leveled off as in controls but TdR kinase continued to increase even as late as 12 hours after infection. In this study the authors concluded that the increase in activity of TdR kinase in infected cells treated with actinomycin D was due to increased stabilization of the preformed enzyme and not to *de novo* synthesis.

3. The Rate of Virus Assembly

Several of the puzzling observations that mesmerized this laboratory for an extended period concerned the rate of appearance of infectious virions in

the infected cells. In one study (ROIZMAN, 1963a), it was found repeatedly that in cells infected with mutant mP and superinfected with mutant MP 3 hours later, the MP progeny of the doubly infected cells appeared 1.4 hours earlier than in singly infected controls. More interesting, however, was the finding that the rate of maturation of the MP mutant followed characteristically the rate of maturation of the mP mutant rather than its own. Thus began a series of studies (ROIZMAN et al., 1963; ROIZMAN, 1963b) whose objectives were to determine what factors affect the rate of virus multiplication. The most interesting observations were that cells, exposed for brief intervals before 6 hours after infection to puromycin or to IUdR, resumed the assembly of virus at the same rate as controls but only after a delay no less than the duration of the exposure to the drug. Cells exposed briefly to the drugs 6 hours or later after infection also recovered afterwards, but the rate of assembly of virus differed from that of controls. On the assumption that viral DNA is made between 4 and 6 hours after infection it was speculated that initiation of DNA synthesis required a critical concentration of enzymes and precursors (ROIZMAN et al., 1963). In retrospect the argument is irrefutable since the critical concentration could consist of a single molecule of DNA polymerase. Additional data to explain these observations have not been forthcoming.

4. Compartmentalization of Herpesvirus Multiplication

As indicated earlier in the text, viral proteins are made in the cytoplasm, viral DNA is synthesized in the nucleus, and viral assembly begins in the nucleus and ends as the virus enters the tubules connecting the nucleus with the extracellular fluid. On the basis of these facts, we may speculate that (i) following uncoating viral DNA is transported (?) into the nucleus, (ii) the DNA is transcribed by cellular DNA dependent RNA polymerase, (iii) the mRNA finds its way into the cytoplasm and directs the synthesis of viral proteins, (iv) the proteins enter the nucleus and begin replication of viral DNA, (v) step 2 is repeated with viral enzymes (?), (vi) step 3 is repeated, (vii) viral coat proteins enter the nucleus and begin assembly and, finally, the assembled nucleocapsid is enveloped by the nuclear membrane and accumulates in the tubules. Two comments should be made concerning the scheme outlined above.

First, were all DNA viruses of the animal kingdom replicating in a similar fashion, it could be argued that compartmentalization of viral multiplication is necessary because of the nature of the eukaryotic cell. It seems reasonable to expect that DNA viruses would be forced to utilize the nucleus — a cellular compartment apparently designed for optimal replication, transcription, and "packaging" of DNA. This, however, is not the case since vaccinia virus multiplies exclusively in the cytoplasm. It should be emphasized that it is not all clear what factors determine whether a virus will multiply in the cytoplasm or in the nucleus or how the virus reaches efficiently the specific site in which it will multiply.

The second comment is that the scheme predicts enormous traffic of informational and structural macromolecules and of virus at the same time

that portions of the nuclear membrane are being utilized to envelope the nucleocapsids. It is not at all clear how this traffic is regulated and what mechanisms determine the destination and transport of mRNA, protein subunits, nucleocapsids, etc. to and across the nuclear membrane.

IV. The Infected Host

> "Read and read again, be not discouraged, but let the existence of so many terrifying diseases that no one can ignore induce you to follow the truth and not the idle chatterers".
>
> PARACELSUS in *Art of Medicine* 1/9, 382

A. General Considerations

The emphasis of the preceding sections was on the virus its structure and reproduction. In this section the emphasis shifts from the virus to the infected host. It is convenient to consider the interaction between the virus and the host at two levels. The first concerns the characteristics of the infected cell. At present we differentiate between infected and noninfected cells by the presence or absence of products specified by the virus. Once the cell makes products specified by the virus it becomes altered. In chronological order, the most common effects of infection on cells grown *in vitro* are (i) changes in shape and structure of the nucleus, (ii) inhibition of host macromolecular synthesis and capacity to divide and (iii) change in the shape of the cell and alterations of the structure of the cellular membrane and of the immunologic specificity of the infected cells. Colloquially, infection is almost uniformly fatal for the cell growing *in vitro*.

The most puzzling, most interesting, and least understood viral-host interaction is that between herpesviruses and the multicellular organisms they infect. The salient feature of this interaction is the ability of the virus to survive in the infected multicellular host even though the host reacts to the infection by making neutralizing antibody. The fundamental conclusion which must be examined is that infection of a cell in the multicellular host is not invariably fatal to the cell.

B. The Unicellular Host

1. Changes in Morphology

The earliest changes following infection are seen in the nucleus. These are (i) aggregation of the chromosomes at the nuclear membranes as shown in Figs. 8B, 8C, (ii) disaggregation of the nucleolus (Figs. 8A, 9A) and (iii) formation of an inclusion. The nucleus itself becomes enlarged and distorted in shape and its membranes frequently fold and fuse.

Between 6 hours after infection and the end of the reproductive cycle, the cell undergoes additional alterations in its general appearance. In sparsely populated cultures of cells growing on glass surfaces, individual infected cells round up and ultimately detach from the glass. In densely populated cultures infected cells may adhere or fuse with adjoining infected or uninfected cells

(ROIZMAN, 1962a). Several reports (STOKER, 1959; STOKER and NEWTON, 1959; VANTIS and WILDY, 1962) have shown that infection can both prevent and abort mitosis. With few exceptions considered further in this section, the consequence of infection is cell death characterized by (i) inability to regain the capacity to synthesize its own macromolecules and (ii) loss of the capacity to multiply. Production of infectious progeny is not a prerequisite for cell death (VANTIS and WILDY, 1962). Numerous investigators reported (i) amitotic nuclear division (SCOTT et al., 1953; KAPLAN and BEN-PORAT, 1959; REISSIG and KAPLAN, 1960; FALKE, 1961; NII and KAMAHORA, 1963) and (ii) chromosome breakage (HAMPAR and ELLISON, 1961, 1963; TANZER et al., 1964; STICH et al., 1964; BENYESH-MELNICK et al., 1964; BOIRON et al., 1966; NICHOLS, 1966; MIKHARLOVA, 1967; WAUBKE et al., 1968; SABLINA and BOCHAROV, 1968) following infection with herpesviruses. These reports are of particular interest in view of the suspicion that herpesviruses may be oncogenic. The published reports and photomicrographs suggest that, following infection with herpes simplex or with pseudorabies, the nuclei become distorted in shape and tend to fragment. The fragments, frequently unequal in size, remain attached to each other. The mechanisms underlying the breakage of chromosomes remain obscure. There is disagreement between RAPP and HSU (1965) and WAUBKE et al. (1968) as to whether virus multiplication is necessary for chromosome breakage even though both agree that the damage is a consequence of a product specified by the virus. The aberrations induced by herpesviruses cannot be readily differentiated with respect to site of occurrence and their nature from those occurring spontaneously or those induced by a variety of mutagenic agents (STICH et al., 1964; HUANG, 1967).

2. Host Macromolecular Metabolism

Information concerning host macromolecular metabolism is available only for cells infected with herpes simplex, pseudorabies and equine abortion viruses. The available information may be summarized briefly as follows:

(i) Host RNA, DNA and protein synthesis (Figs. 3—5) are inhibited during the first 3 to 5 hours after infection (ROIZMAN and ROANE, 1964; ROIZMAN et al., 1965; SYDISKIS and ROIZMAN, 1966, 1967; AURELIAN and ROIZMAN, 1965; HAY et al., 1966; O'CALLAGHAN et al., 1968a; FLANAGAN, 1967; BEN-PORAT and KAPLAN, 1965). The inhibition of the synthesis of host macromolecules coincides with the disaggregation of the nucleolus, disaggregation of the polyribosomes and aggregation of chromosomes at the nuclear membrane (SYDISKIS and ROIZMAN, 1966, 1967; SCHWARTZ and ROIZMAN, manuscript in preparation).

(ii) There is no test capable of measuring precisely the ability of the virus to differentiate in its inhibition between the synthesis of its own and host macromolecules. A pragmatic test is to compare viral yields from cells infected at different multiplicities of infection, the assumption being that, if the virus cannot efficiently differentiate between its own and host macromolecular synthesis, increasing the multiplicity of infection should decrease virus yield.

The rate of inhibition of HEp-2 protein (SYDISKIS and ROIZMAN, 1968) and RNA synthesis (WAGNER and ROIZMAN, manuscript in preparation) may be accelerated by increasing the multiplicity up to approximately 100 p.f.u. of herpes simplex virus per cell. Increasing the multiplicity beyond that amount does not further accelerate the inhibition of the host. Virus yield is independent of the multiplicity in the range of 10—1000 p.f.u./cell. It seems reasonable to conclude that, unlike adenoviruses (LEVINE and GINSBERG, 1967, 1968), herpesviruses are able to differentiate between host and viral macromolecular biosynthesis.

(iii) The inhibition of host DNA and protein synthesis appears to be total. There is some suggestion that the inhibition of host RNA synthesis is selective it is based on the observation that the synthesis of 4S RNA declines more slowly than that of ribosomal RNA (WAGNER and ROIZMAN, manuscript in preparation).

(iv.) It is not clear whether the inhibition of protein synthesis, for example, is the cause or consequence of the inhibition of cell nucleic acid synthesis. For heuristic if not factual reasons, it seems profitable to consider that the synthesis of each macromolecule is inhibited independently of the other two. The observation that herpes simplex virus inhibits protein synthesis more rapidly than actinomycin D (SYDISKIS and ROIZMAN, 1967) does not detract from this view.

(v) The efficiency of the inhibitory process appears to be determined by the host. The process is very efficient in HEp-2 cells infected with herpes simplex virus at relatively low multiplicities of infection. Much higher multiplicities of infection are required to produce equivalent inhibition of the macromolecular syntheses of dog kidney cells by the same virus (AURELIAN and ROIZMAN, 1965).

(vi) The mechanisms by which viruses inhibit the host macromolecular synthesis are unknown. Reports concerning the nature of the products responsible for the inhibition are contradictory. BEN-PORAT and KAPLAN (1965) demonstrated that puromycin (20 μg/ml) decreased the rate of inhibition of host DNA synthesis in rabbit kidney cells infected with pseudorabies virus. On the other hand NEWTON (1968) reported that ultraviolet light inactivated herpes simplex virus was effective in blocking host DNA synthesis and moreover, the effect of the virus could not be abolished by chemical inhibitors of protein synthesis. Herpes simplex virus loses its capacity to disaggregate HEp-2 cell polyribosomes and inhibit protein synthesis following ultraviolet light-irradiation (SYDISKIS and ROIZMAN, 1967). Whereas actinomycin D, p-fluorophenylalanine, and 6-azauridine prevented the disaggregation of host polyribosomes in dog kidney cells abortively infected at relatively high (1000 p.f.u./cell) multiplicities of infection, the drugs were ineffective in HEp-2 cells infected at low multiplicities (SYDISKIS and ROIZMAN, 1967). The difference in the effectiveness of the drugs was not due to inherent differences between HEp-2 and DK cells since actinomycin D was ineffective in preventing the disaggregation of host polyribosomes in DK cells infected with a

herpes simplex mutant (MP$dk^{+}sp$) capable of growing in these cells. The results of various studies summarized elsewhere (AURELIAN and ROIZMAN, 1965; SPRING et al., 1968; SYDISKIS and ROIZMAN, 1967) indicate that infection of DK cells with the MPdk^{-} strain of herpes simplex virus aborts because one or more functional proteins specified by the virus are not made. One hypothesis which may account for these observations may be stated as follows: In productive infection of permissive cells (HEp-2), the inhibitor of protein synthesis functions catalytically and is highly efficient. Even trace amounts of this substance made in the presence of chemical inhibitors or of interferon are sufficient to inhibit the host. In abortively infected cells the inhibitor of protein synthesis is less efficient, more inhibitor molecules are necessary to shut off host protein synthesis and hence any interference by drugs with either the synthesis of the inhibitor or its function tends to prevent the inhibition of the host.

This brief account of the inhibition of host functions reflects the amount of available data but does little justice to the subject. The significance of the phenomenon stems from two considerations. First, the inhibitor induced in the cell by the virus must be able to differentiate between cellular and viral polyribosomes and between viral and cellular DNA with respect to both replication and transcription. There is at the moment no evidence that infected cells contain new or different nucleotides or new sRNA's differing in genetic coding properties from those of uninfected cells. If further studies bear this out, it would necessarily follow that the inhibitor functions by interacting directly with host templates. This conclusion presents two difficulties. First, to preferentially react with host templates, there must be differences between viral and cellular nucleic acids. Second, it is difficult to visualize the evolutionary development of a macromolecule with greater affinity for host templates than its own, particularly in view of the fact that herpesviruses multiply readily and effectively in a wide variety of species throughout the animal kingdom. It should be pointed out that, even though at present there is no evidence that host and viral nucleic acid differ with respect to some structure of invariant nucleotide sequence which could serve for recognition of self and nonself, some invariant differences must exist since the cell stimulated by interferon is also capable of differentiating between viral and cellular macromolecular synthesis. If, as currently suggested by MARCUS and SALB (1966), LEVY and CARTER (1968) and by CARTER and LEVY (1967, 1968), ribosomes treated with interferon differentiate between viral and cellular messenger RNA, it follows that there is a difference between corresponding cellular and viral RNA involved in protein synthesis and that this difference may be the basis for cellular exclusion of viral protein synthesis and vice-versa. As indicated elsewhere (ROIZMAN and SPEAR, 1969) it also follows that (i) in the cell not stimulated with interferon the protein synthesizing machinery does not discriminate between viral and cellular RNA, (ii) interferon imposes a restriction on the cell and (iii) from the temporary nature of the restriction it may be deduced that it is not in the best interest

of the cell or of the organism to restrict its translation of RNA to molecules bearing a specific cognitive structure.

The second consideration relates not so much to the nature of the inhibitor as to the fact that herpesviruses contain genetic information for the inhibition of the host. It is not very clear why viruses have acquired, retained, and expressed the capacity to inhibit the host. The only available data pertinent to the problem raised here would seem to indicate that inhibition of host macromolecular synthesis is a prerequisite for virus multiplication. This conclusion is based on the observation that the MP*dk*$^-$ strain of herpes simplex virus multiplies and effectively inhibits cells of human derivation but does not produce infectious progeny and does not effectively inhibit DK cells, largely because as indicated elsewhere in this section, some proteins specified in DK cells malfunction. The significant finding is that in DK cells viral DNA and proteins are synthesized only in cells infected at a multiplicity sufficiently high to inhibit the host. At low multiplicities of infection the cell makes interferon only (AURELIAN and ROIZMAN, 1965). The data would seem to indicate that (i) host response to infection and inhibition of host macromolecular synthesis are competing processes initiated on infection and that (ii) at low multiplicities of infection the cells attain the upper hand only because the amount of effective inhibitor specified by the virus is insufficient to inhibit the host in time to prevent it from inhibiting the virus (AURELIAN and ROIZMAN, 1965; SYDISKIS and ROIZMAN, 1967).

3. Alteration in Immunologic Specificity

The studies on the immunologic specificity of cells infected with herpesviruses was prompted by the observation that viruses frequently alter the social behavior of cells (ROIZMAN, 1962a). In general the shape, adhesiveness, and social behavior of the cell are inherited by properties determined by the structure of the cellular membrane. The observation that viruses modify these properties implies that the structure of the cytoplasmic membrane has become altered. The evidence that infected cells acquire a new immunologic specificity was obtained with the aid of a test based on the observation that viruses fail to multiply in somatic cells injured by antibody and complement (ROIZMAN and ROANE, 1961b). In practice, cells are infected with herpes simplex virus, suspended, washed and incubated at 37°C with appropriate amounts of antibody and complement. After 1 hour the cells are diluted in an appropriate medium and seeded on monolayer cultures of HEp-2 cells. The survivors produce plaques whereas injured cells do not. Both antibody and complement are required for immune injury; antibody alone or complement alone is ineffective. The sole function of infected cells is to provide a measurement of the fraction of the test population that remains viable after exposure to antibody and complement. The sensitivity of the test stems from the fact that very few cells are needed since nearly every infected cell produces a plaque. The assay was initially standardized with 2 hour infected cells and antibody against uninfected cells. Pertinent here are the findings that the

immunologic specificity of 2 hour infected cells could not be differentiated from that of uninfected cells and that the differentiation between "viable" and "killed" cells was not affected by the type of monolayer culture used for enumeration of infective centers.

The alteration of immunologic specificity after infection was demonstrated in tests employing 20—24 hour infected cells and rabbit sera prepared against infected cells (ROANE and ROIZMAN, 1964b). The tests showed that complement and unabsorbed anti-infected cell serum precluded the formation of plaques by 2, 24 and 48 hour infected cells. However, following absorption with uninfected cells, the serum and complement precluded the formation of plaques by 24 and 48 hour infected cells only; the absorbed serum was not effective against 2 hour infected cells. Clearly, the 24 and 48 hour infected cells contain on their surface one or more antigens absent in uninfected cells. The conclusion that the membranes of infected cells become altered with respect to structure and immunologic specificity was corroborated in a study by WATKINS (1964) showing that HeLa cells infected with the HFEM strain of herpes simplex acquire "stickiness" for sheep erythrocytes sensitized with rabbit anti-sheep erythrocyte serum. The adhesion of sensitized erythrocytes to the infected cells could be abolished by exposing the infected HeLa cells to anti-viral serum. Normal rabbit serum and rabbit anti-sheep erythrocyte serum failed to prevent the adhesion of the sensitized erythrocytes to infected HeLa cells.

The weight of the evidence favors the hypothesis that the new antigen is a structural component of the viral envelope. The evidence consists of the following findings: (i) Absorption of serum with partially "purified" virus removes both neutralizing and cytolytic antibody (ROANE and ROIZMAN, 1964b). However, the significance of this observation is limited by the fact that the most purified virus preparations were not free of host antigens. (ii) Assays of hyperimmune sera prepared against a variety of antigens extracted from infected permissive and nonpermissive cells showed an excellent correlation between neutralizing and cytolytic titers (ROIZMAN and SPRING, 1967). (iii) DK cells abortively infected with MP*dk*$^-$ virus produce naked nucleocapsids only; envelopment does not take place (SPRING et al., 1968; SPRING and ROIZMAN, 1967). Rabbit hyperimmune sera produced against extracts of abortively infected DK cells lack both neutralizing and cytolytic antibody (ROIZMAN and SPRING, 1967).

4. The Social Behavior of Dispersed Cells in Culture

a) *Description and Applications.* Numerous laboratories have reported the isolation of herpes simplex, pseudorabies and herpes B virus strains differing with respect to their effects on cells (TOKUMARU, 1957; GRAY et al., 1958; HOGGAN and ROIZMAN, 1959; HINZE and WALKER, 1961; FALKE, 1961; NII and KAMAHORA, 1961; KOHLHAGE and SIEGERT, 1962; SCHNEWEISS, 1962b; KOHLHAGE, 1964; WHEELER, 1964; KOHLHAGE and SCHIEFERSTEIN, 1965; EJERCITO et al., 1968; SCHIEK, 1967; SCHIEK and SCHNEWEISS, 1967). Herpes

simplex virus strains were recently (EJERCITO et al., 1968) classified into four groups i.e., (i) strains causing rounding of cells but no adhesion or fusion, (ii) strains causing loose aggregation and rounded cells, (iii) strains causing very tight adhesion of rounded infected cells, and (iv) strains causing polykaryocytosis. The viruses comprising each group may differ in fine detail with respect to their effects on cells. Thus, polykaryocytes induced by various strains of herpes simplex virus differ in size and morphology (ROIZMAN and AURELIAN, 1965; KOHLHAGE, 1964; WHEELER, 1964).

A plaque assay of herpes simplex virus based on the alteration in the social behavior of infected cells has been described (HOGGAN et al., 1950; ROIZMAN and ROANE, 1961a, 1963). Development of the assay was facilitated by the observation of BLACK and MELNICK (1955) that the spread of herpes B virus from cell to cell by direct extension is not blocked by neutralizing antibody. In practice HEp-2 cells grown in monolayer cultures are exposed to appropriate dilutions of virus, then overlayed with medium containing human pooled γ globulin as the source of antibody. Plaques develop after 40—48 hours of incubation at 37°C. To simplify the enumeration of plaques, the liquid overlay is removed, the cells are fixed with methanol, stained with Giemsa, then air dried. The plaque morphology and cell interaction caused by some strains so different as to permit simultaneous assays of artificial mixtures of two or more mutants. Provided the number of plaques is not excessive, there is no reciprocal interference between the strains and the plaque counts are proportional to the concentration of virus in the artificial mixture. Studies based on the use of artificial mixtures have revealed that viruses differing with respect to their effects on the behavior of cells frequently differ also with respect to (i) buoyant density in CsCl, (ii) stability at 40°C (iii) patterns of elution from brushite columns and (iv) immunologic specificity (ROIZMAN and ROANE, 1963; ROIZMAN and AURELIAN, 1965; KOHLHAGE, 1964; EJERCOITO et al., 1968; SCHIEK, 1967).

b) *The Basis for the Alteration in the Social Behavior of Infected Cells.* The mechanism by which herpesviruses alter the social behavior of infected cells is largely unknown. The little that is known is based on a few experiments and much deductive reasoning and covers three points as follows:

(i) The structure of the cell which interacts with other cells and determines the adhesiveness is the cytoplasmic membrane. It follows that changes in the social behavior of cells invariably reflect corresponding changes in function, structure and chemical composition of cellular membranes.

(ii) The alterations in social behavior induced by herpesviruses are also induced by other viruses and by chemicals such as certain lipids, lipophilic substances and parathyroid hormone (ROIZMAN, 1962a). Moreover, the alterations induced by the same virus in different cell lines may vary considerably (ROIZMAN and AURELIAN, 1965; ROIZMAN, 1962a). On the basis of these observations it must be concluded that the cell responds in a similar fashion to a variety of dissimilar agents in a specific and reproducible manner.

(iii) As indicated in Section II, D, the hypothesis which fits best the available data is that cellular membranes must be modified by products specified by the virus in order for the virion to become enveloped. The hypothesis envisions that the product inducing the modification in the membranes is also responsible for the immunologic specificity of the virus and of the infected cells and for the alteration in the cytoplasmic membranes resulting in an altered social behavior of the infected cells. One prediction of the hypothesis has been fulfilled. Thus mutations in the virus resulting in an altered structure of the envelope confer a new immunologic specificity to the virus and induced in infected cultures a new pattern of social behavior (ROIZMAN and AURELIAN, 1965; EJERCITO et al., 1968). Ultimately, however, it will be necessary to show that a defined structural component of the envelope is, in fact, responsible for the alteration of the social behavior of infected cells.

C. The Multicellular Host

1. Persistence of Virus in the Host

Herpesviruses have a well deserved reputation of being ubiquitous and capable of persisting in the host they infect. Most of the information concerning the capacity of the virus to persist consist of observations on recurrent herpes simplex infection of man. Man is infected with herpes simplex virus between 6 months and 5 years of age. About 1 per cent of those infected suffer mild or severe illness which runs its course in 1—3 weeks. For the rest the initial infection is inapparent or cannot be differentiated from other infectious episodes of infancy and childhood. However, as many as 75 per cent of those who have contracted primary infections (as evidenced by the presence of anti-viral antibody in their blood) are afflicted at some time during their lives with recurrent herpetic eruptions. The unique and puzzling feature of herpesvirus infection of man is that individuals subject to recurrent herpetic episodes can often predict the recrudescences accurately; the lesions appear following a specific physical or emotional provocation. It is now generally accepted that (i) following primary infection the virus is harbored in an inapparent form at some particular site and that (ii) specific stimuli associated with physical (reviewed by ROIZMAN, 1965c) and emotional (BLANK and BRODY, 1950) provocations of the host cause the virus to manifest itself in the form of typical herpetic lesions (ROIZMAN, 1965c; TERNI, 1965). The mechanisms by which the virus survives in the immune host in the interim between recrudescences are laregly obscure. Two hypotheses have been proposed (ROIZMAN, 1965c) to account for this behavior: (i) the virus multiplies at a reduced rate in some tissue and (ii) the reproductive cycle is arrested soon after infection and before the synthesis of proteins specified by the virus. Both hypotheses envision that biochemical responses to physical or to emotional provocations either increase the rate of multiplication (Hypothesis 1) or remove the inhibition (Hypothesis 2). At present there is too little data to support either hypothesis. Some of the more tantalizing observations and reports are that (i) herpesviruses have not been isolated from the site of the recurrences in

the interim between recrudescences (RUSTIGAN et al., 1966; CORIELL, 1963; M. TERNI, personal communication). (ii) Viruses isolated from recurrent lesions on different parts of the body of the same individual may differ with respect to their effects on the social behavior of cells (M. TERNI, personal communication). (iii) It has been claimed by ASHE and SCHERP (1965) that viruses isolated during successive recurrences vary with respect to immunologic specificity. Cumulative changes in the antigenic properties of herpes simplex virus have also been reported to occur in persistently infected cultures of Chinese hamster cells (HAMPAR and KEEHN, 1967). However, in permissive cell lines the viruses are genetically stable. (iv) There is considerable evidence (reviewed by ROIZMAN, 1965, and by TERNI, 1965) suggesting that the virus is associated with or controlled by sensory nerves. (v) Attempts to arrest the reproductive cycle by manipulating the physiologic state of the cell in vitro have failed (ROIZMAN, unpublished studies). However, as reviewed by TERNI (1965), long term herpesvirus carrier cell lines are readily established particularly with primary cell lines.

2. Herpesviruses and Cancer: Known Associations

Herpesviruses are not known to induce tumors in hosts they do not naturally infect except with the aid of carcinogens (TANAKA and SOUTHAM, 1965). Herpesviruses are associated with three naturally occurring tumors, i.e., with the lymphoma occurring chiefly in Africa and described by BURKITT (1958), with Marek's disease of fowl (WIGHT et al., 1967) and with Lucké adenocarcinoma of frogs (FAWCETT, 1956; LUNGER, 1964). Of the three, Burkitt lymphoma and Lucké adenocarcinoma have been studied most extensively.

The virus present in Burkitt lymphoma cells is not readily apparent in the tumor cells in situ; it becomes readily apparent, however, in cells removed from tumors and grown in culture, particularly in media and at temperatures suboptimal for cell growth (HENLE and HENLE, 1968). The presence of virus is manifest by (i) enveloped and unenveloped nucleocapsids detected by electron microscopy (ii) intracellular antigen visualized in acetone-fixed cells with conjugated antisera (HENLE and HENLE, 1966a, b) (iii) membrane-bound antigen visualized by staining live cells with conjugated antibody (KLEIN et al., 1966, 1967a). In general, there appears to be a good correlation between presence of virus in the cell and the two types of antigen (intracellular and cell surface) detected by immunofluorescence (HENLE and HENLE, 1966a, b; HINUMA et al., 1967; KLEIN et al., 1968a). The crux of the problem is the relationship of the virus and the lymphoma. There are no suitable experimental animals; in fact the only cells which are readily susceptible to infection with cell-free virus are Burkitt tumor cells rendered free of virus by rapid passages in enriched media at 37°C. In evaluating the relationship between the virus and the tumor three observations are of particular interest: (i) the same or a closely related virus may be responsible for infectious mononucleosis — an acute infectious disease of young adults (HENLE et al., 1968; KLEIN et al., 1968a). (ii) The lymphomas in African children occasionally regress. Regression

correlates with the development of anti-viral antibody (KLEIN et al., 1967b, 1968b, c). (iii) Chemotherapy has been singularly successful against Burkitt tumors. Some of the drugs that have been used are Orthomerphalan, Cytoxan and Cytosine Arabinoside. The results of chemotherapy appear to correlate with the patients capacity to produce antibody against the virus as measured in membrane immunofluorescence tests (BURKITT, 1967; CLIFFORD et al., 1967).

The herpesvirus associated with the Lucké adenocarcinoma is present in the tumors of frogs collected during the Winter but not during the Summer months. However, virus particles readily develop in tumors of frogs collected during the Summer and refrigerated for several weeks (RAFFERTY, 1967). TWEEDELL (1967) succeeded in inducing tumors in tadpoles following injection with cell free extracts of tumors containing virus. Subsequently, MIZELL (1969) and TOPLIN et al. (1969) succeeded in inducing tumors in tadpoles with material consisting of partially purified enveloped nucleocapsids extracted from a tumor. One of the major problems plaguing amphibian virology is the presence of numerous viruses, parasites, etc., in kidneys of frogs (GRAVELL et al., 1968; GRANOFF et al., 1969). It seems clear that the demonstration of a causal relationship between the herpesvirus and the Lucké adenocarcinoma requires the induction of tumors by highly purified preparations of the virus.

3. Herpesviruses and Cancer: Prospective Association

There have been many suggestions that herpesviruses notably herpes simplex viruses cause cancer in man. Some of the clues which have lead to this suggestion are based on (i) the effect on cells in culture and (ii) occurrence of tumors at the site of recurrent infections.

The clues derived from observations on infected cells in culture consist of reports that herpesviruses cause amitotic nuclear division, chromosome breakage and proliferation of cells. As pointed out in Section IV, B, 1, fragmentation of nuclei is easily mistaken for amitotic nuclear division. Both terms describe the same phenomenon, but the implications are as different as day and night! Likewise, as pointed out in the same section, there is no evidence that the chromosome aberrations reported in nearly a dozen papers are induced at a specific site characteristic of herpesviruses. Perhaps most unfortunate is the perpetuation of a myth that some virus strains cause proliferation of infected cells. The myth derives its origin from a paper by GRAY et al. (1958) who designated one of their strains as "proliferative" and reported that cells in cultures infected with this virus increase in number. The same laboratory subsequently reported this to be untrue (SCOTT et al., 1961). Despite numerous denials published since then (the latest one by SAKSELA and VAHERI appeared in 1968) the term proliferative seems to have stuck. The problem in part is that in cultures infected at relatively low multiplicities of infection and particularly in the presence of anti-viral antibody the infected cells adhere very tightly to each other and form clumps on the surface of the monolayer cell culture superficially resembling foci of rapidly growing transformed cells.

The clues based on epidemiologic studies are more tantalizing. There are two series of studies as follows: (i) An association between recurrent herpetic infection and squamous carcinoma of the lip has been reported by two investigators i.e., by WYBURN-MASON (1957) and by KVASNICKA (1963, 1965). The first reported a series of case histories. The other reported studies involving many patients. Both investigations conclude that frequent recurrent lesions over a long time at one site ultimately cause malignancy in a manner as yet unknown. These studies are difficult to evaluate for two reasons. First, in the affected individual the fever blisters recurs on the same area of the face but not in the topologically identical place. The greatest difficulty is that squamous cell carcinoma of the lip is rather rare! The selection of suitable populations for prospective studies would be difficult and the surveilance itself would be very costly. The second series of studies concerns the association of herpes simplex and cervical cancer. There is adequate evidence that there are at least two types of herpes simplex viruses infecting man. The viruses differ with respect to physical, immunologic, and certain biologic properties (SCHNEWEISS, 1962a; PLUMMER, 1964; DOWDLE et al., 1967; PLUMMER et al., 1968; EJERCITO et al., 1968). One of the two causes predominantly infection of the genitalia transmitted by sexual contact (NAHMIAS et al., 1967; DUXBURY and LAWRENCE, 1959; BEILBY et al., 1968). On epidemiologic grounds at least two laboratories have suggested that the genital strain might be responsible for cervical cancer in women (JOSEY et al., 1968; NAHMIAS, 1968; RAWLS et al., 1968). However, the results of the epidemiologic studies relating cervical cancer with genital herpes infection are not entirely convincing. Moreover, cervical cancer occurs most frequently in a population characterized by sexual permissiveness, promiscuity, and poor personal hygiene (BEILBY et al., 1968). The same population harbors preferentially (but not by choice) selected bacteria and parasites as well as the genital strain of herpes simplex virus. Obviously it remains to be seen whether the virus alone, to the exclusion of all other vaginal inhabitants, is the oncogenic agent.

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Growth Patterns of Antibody-Forming Cell Populations[1]

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With 15 Figures

Contents

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I. Introduction

A comprehensive review of the proliferative events involved in the antibody response will not be attempted here in the classical sense. Rather, we shall take an unorthodox approach and discuss, in relation to various models, the current data on the antigen-triggered exponential rise in antibody-forming cells. There are several reasons for taking this approach. 1. In recent years there have been several comprehensive reviews (DUTTON, 1967; MAKINODAN and ALBRIGHT, 1967; UHR and FINKELSTEIN, 1967), symposia (Symposium on Molecular and Cellular Basis of Antibody Formation, 1965; Cold Spring Harbor Symposia, 1967), and workshops (Midwinter Conference of Immunologists, 1968; Gordon Research Conferences, Immunochemistry and Immunobiology, 1968; Southeastern Workshop of Immunology, 1968) covering this subject, but critical evaluation of models has been lacking. 2. In view of recent technological advances in immunology and data analysis, most models are now testable and therefore should be discussed. 3. By approaching this problem in terms of model analysis, we hope that quantitative data on antigen-triggered differentiation (transformation, proliferation, and death) can be obtained. We can then focus our attention on the more complex and fascinating problem of homeostasis in the immune response at the cellular level. 4. Increasing numbers of immunochemists, clinical immunologists, and even non-immunologists are becoming interested in this problem because of its broad fundamental and clinical implications. We believe that individuals of such diversified backgrounds can become proficient in this subject more quickly after exposure to model analysis. With these readers especially in mind, we shall include in this review brief treatments of the histological changes induced by antigen in lymphoid organs and of the cellular events involved in initiating the antibody response.

II. Histological Changes in the Spleen

A. Changes in Situ

It has long been known that following antigenic stimulation of adults, cells in the spleen and lymph nodes undergo transformation and proliferation before detectable amounts of antibody appear in the blood (e.g., see FAGRAEUS, 1948; MCMASTER, 1953). However, a detailed analysis based on close-interval observations of the sequential events was lacking until about a decade ago (WHITE, 1960; CONGDON and MAKINODAN, 1961; LANGEVOORT, 1963; FLIEDNER et al., 1964; HANNA, 1965; CRADDOCK et al., 1967; MCMASTER and FRANZL, 1968).

Before considering these events, brief comment should be made on the main features of the spleen. The spleen is divided into the white pulp and the red pulp (see Fig. 1). The white pulp forms an uneven sheath of lymphoid tissue enveloping the central arterioles. Expanded areas of this periarteriolar sheath constitute lymphocyte masses, often described as lymphatic nodules, which frequently contain germinal centers. At the periphery of a lymphatic

nodule is a narrow region consisting of marginal sinuses and a marginal zone, which delineates the boundary between white pulp and red pulp[5]. The red pulp consists of venous sinuses and splenic cords, the tissue between the sinuses. The areas of the lymph node comparable to the white pulp and red pulp of the spleen are the cortex and the medulla, respectively.

The antigen-triggered cellular events in the spleen and the lymph nodes are comparable. Furthermore, the events in the primary response do not

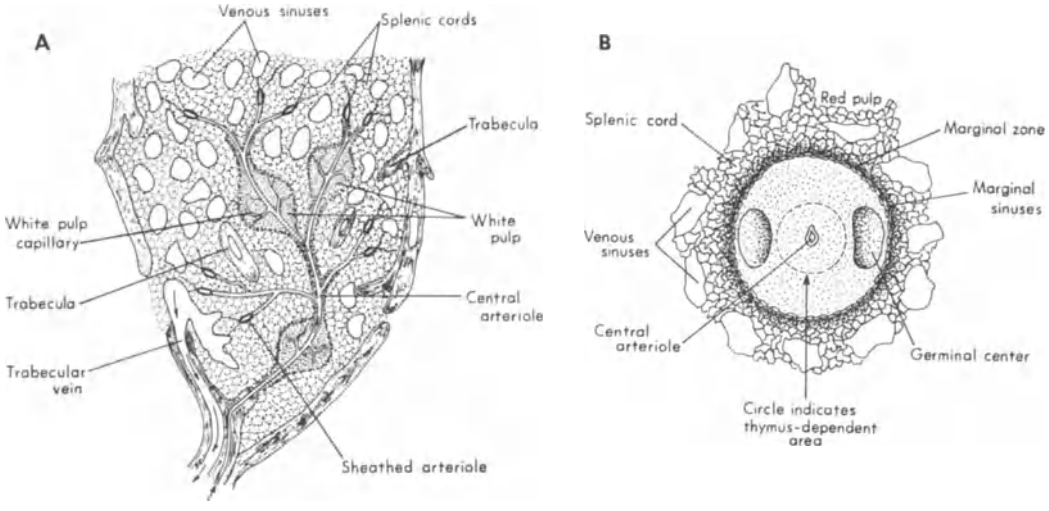


Fig. 1 A and B. Diagram of the overall architecture of the spleen (A) and a cross section of a lymphatic nodule (B)

differ *qualitatively* from those in the secondary response. Hence, we shall cite only those events occurring in the spleen of an individual undergoing a primary response. They are as follows:

1. Germinal centers undergo hyperplasia within 2 hours after stimulation with high antigen doses.

2. The germinal centers lose their architecture during the next 22 hours, finally disappearing completely. This process has been called "germinal center dissociation". During the dissociation period, blast cells[6] increase in number and are randomly scattered throughout the nodules, eventually becoming concentrated in the immediate periarteriolar region 24 hours after antigenic stimulation.

3. On day 2, the dissociation of germinal centers persists; blast cells now appear frequently as clusters in the marginal zone and have begun to infiltrate the red pulp.

4. On day 3, the proliferation of blast cells in the red pulp is very marked and these cells are clearly associated with clusters of plasma cells. On the same day, the germinal centers reappear and undergo hyperplasia.

[5] It has been reported (SNOOK, 1964) that in the rat spleen, erythrocytes and particulate matter pass from white pulp capillaries into the marginal sinuses and then through pores into the mesh of the marginal zone, from which they flow outward into the red pulp.

[6] These cells are also known as large pyroninophilic cells (HANNA, 1965), hemocytoblasts (FAGRAEUS, 1960), plasmablasts (FAGRAEUS, 1948; LANGEVOORT, 1963), and immunoblasts (DAMESHEK, 1962; MOVAT and FERNANDO, 1964).

5. By 4 weeks there is a return to normal architecture, and the rate of return appears to be inversely related to the amount of antigen injected.

Germinal centers are absent in certain animals; for example, germfree mice (HANNA et al., 1969). Obviously, early hyperplasia and dissociation of germinal centers do not occur in their spleens. Nevertheless, they can respond as well as those whose spleens contain preexisting germinal centers (TENNANT et al., 1965; BOSMA et al., 1967). In germfree mice the first proliferative event

Table 1. *Contribution of hemolysin plaque-forming cells to increase in spleen cell number*[a]

	No. Cells per spleen (in millions)	Net increase in cells per spleen (A) (in millions)	Net increase in PFC per spleen (B) (in millions)	B/A (%)
Mean (95% confidence limits)				
Before Ag				
$n = 71$	201.65			
	(200.92, 201.74)	71.32	0.5—1.0	0.7—1.4
After Ag		(71.17, 71.46)		
(3—4 days)	272.65			
$n = 118$	(272.09, 273.20)			

[a] Young adult mice were injected intravenously with 100 million sheep erythrocytes and sampled 3—4 days later (PERKINS et al., unpublished).

occurs in the area immediately surrounding the central arterioles and is associated with the appearance of 19S (IgM) antibody-forming cells. It would appear, therefore, that the germinal center hyperplasia and dissociation observed in conventional animals during the first 24 hours after antigenic stimulation is not essential for the initiation of the 19S (IgM) antibody response. However, the reappearance of germinal centers and their secondary hyperplasia may be associated with the appearance of 7S (IgG and IgA) antibody.

All cells involved in the early periarteriolar proliferative event may not necessarily reflect stages in differentiation leading to the appearance of specific antibody-forming cells. The reason for this remark is that most of the antigen-triggered transformation and proliferation events are manifestations of cells *not* engaged in the production of antibody-forming cells. For example, we have found that at the end of the transformation and proliferation events there was a net increase of about 70 million nucleated cells in the spleen, of which not more than one million were hemolysin antibody-forming cells (see Table 1). It should be apparent that more supportive data are needed before one can begin to make "educated guesses" as to where in the spleen the early events of differentiation of immunocompetent cells occur, what cells are involved, and how they interact. Some of these subjects are subsequently discussed.

B. Effect of Immunosuppressive Insults

Studies on the effect of insults have contributed immensely to an understanding of cellular differentiation of immunocompetent cells. Basically there are three categories of insults: physical, chemical, and surgical.

The most extensively employed physical insult is ionizing radiation (for comprehensive reviews, see TALIAFERRO et al., 1964; MAKINODAN, 1966; NOSSAL, 1967). In retrospect, it can be seen that radiation immunologists were the first to suggest that immunocompetent cells resided in the white pulp and that proliferation was an integral part of the overall differentiation process; that is, in 1903 HEINEKE observed the high radiosensitivity of the white pulp, and in 1908 BENJAMINI and SLUKA showed that the cellular events occurring during the first 72 hours after antigenic stimulation were extremely radiosensitive. Subsequent studies (MAKINODAN et al., 1962; CELADA and CARTER, 1962; SMITH and VOS, 1963; KENNEDY et al., 1965) clearly established that the radiosensitivity indices of the early cellular events were comparable to those characteristic of proliferating mammalian cells; i.e., the D_0 dose was about 75 R and the extrapolation number about 3. In contrast, the terminal, mature antibody-forming cells were shown to withstand X-ray doses as high as 10,000 R and to synthesize antibodies as efficiently as unirradiated cells for several days (MAKINODAN et al., 1967; VANN and MAKINODAN, 1969; SADO, 1969).

Studies on the effect of chemical insults are also numerous (for comprehensive reviews, see DUTTON and PEARCE, 1962; MAKINODAN et al., 1965; SCHWARTZ, 1967). We shall limit our discussion to only one of these agents to emphasize how such an agent can be used to establish the time at which the earliest, essential proliferative event occurs after antigenic stimulation. This was done by PERKINS et al. (1968), who injected mice with vinblastin, a mitotic poison, at various intervals after antigenic stimulation and assessed its effect in terms of the number of antibody-forming cells in the spleen. Their results showed that the essential proliferative event did not occur until more than 12 hours after antigenic stimulation.

With regard to surgical ablation, the most widely investigated during the past decade has been thymectomy and its effect on immune competence. There have been several reviews on this subject (GOOD and GABRIELSON, 1964; MILLER, 1966; MILLER and OSOBA, 1967), and readers are referred to them for comprehensive information. Suffice it here to say that the thymus is essential for the development of immune competence and that its removal during neonatal life can cause a fatal immunological defect. In the spleen this defect is associated with the depletion of lymphoid cells immediately surrounding the central arterioles. Thus, this area has been referred to as the "thymus-dependent" area (PARROT et al., 1966; DE SOUSA and PARROT, 1967).

All these studies suggest that the proliferative event occurring 12 hours after antigenic stimulation is essential for the production of antibody-forming cells. They further suggest that cells of thymic origin may be involved in this early proliferative event.

C. Reconstitution Studies

Two methods have been employed to reconstitute the spleen after radiation-induced depletion. One is called "endocloning" reconstitution, performed primarily by SIMIĆ and his colleagues (SIMIĆ, 1966; SIMIĆ and PETROVIĆ, 1967). In essence, the exteriorized spleen of a lead-shielded animal is virtually "sterilized" with 10,000 R of X-rays immediately after antigenic stimulation. This destroys practically all of the lymphoid cells, but the reticuloendothelial network remains intact, at least temporarily, and allows recolonization with competent cells from the circulation. There are several advantages of this system. 1. The architecture of the depleted spleen is not as complex as that of the unirradiated spleen. 2. The cells recolonizing the depleted spleen are not manipulated by the investigator. Indeed it has been reported that hematopoietic stem cells are injured by such manipulation and that the rate of their differentiation may be altered (LAJTHA, 1966). 3. The experimental animals whose spleens were X-rayed produced as much, if not more, antibody than the unirradiated animals. Histological studies showed that within 12 hours small lymphocytes or lymphocyte-like cells colonize areas immediately surrounding the central arterioles. These cells then appear to undergo transformation into blast cells and migrate out to the periphery and into the red pulp, where plasmacytopoiesis occurs. At the time of maximum plasma cell formation, numerous germinal centers are activated. These studies show that the progenitors of antibody-forming cells are not the fixed reticular or endothelial cells, a view that has been held by many (FAGRAEUS, 1948; MARSHALL and WHITE, 1950; WISSLER et al., 1957; BERNHARD and GRANBOULAN, 1960), but are blood-derived.

The second method is referred to as cell transfer or "exocloning" reconstitution. In contrast to endocloning reconstitution, the whole animal is given a lethal dose of ionizing radiation, generally after thymectomy. These animals can live without further treatment for a week. An animal exposed to a lethal dose of ionizing radiation is immunologically unresponsive and therefore serves as an excellent *in vivo* culture for lymphoid cells (e.g., see review by MAKINODAN et al., 1960). Thus the experimenter can infuse various types and numbers of dispersed cells into these "*in vivo* test tubes". This type of study will be discussed in more detail in the subsequent section, because it has been one of the key approaches to the establishment of our current view that at least two cell types are needed for the initiation of the antibody response. Only the histological findings will be considered here. One of the first definitive studies demonstrating the need for interaction of thymus-derived and bone marrow-derived cells in the reconstitution of a depleted spleen was performed by DAVIES, KOLLER, and their colleagues (DAVIES et al., 1966, 1967; KOLLER et al., 1967). These investigators demonstrated that in response to antigenic stimulation the thymus-derived cells proliferated before detectable antibody-forming cells appeared. They also showed that the thymus-derived cells were not the precursors of antibody-forming cells. This latter observation was confirmed by the brilliant series of studies performed by MILLER and MITCHELL

and their collaborators (MITCHELL and MILLER, 1968b; MILLER and MITCHELL, 1968; NOSSAL et al., 1968b, MARTIN and MILLER, 1968). These investigations suggest that the initial antigen-triggered proliferative event in the periarteriolar area may reflect either the interaction of antigen with the thymus-derived, antigen-reactive cells or the interaction of antigen-stimulated, thymus-derived cells with the precursors of antibody-forming cells.

D. Fate of Antigen

Studies on the localization of antigen have been based on immunofluorescence techniques (KAPLAN et al., 1950; HILL et al., 1950; COONS et al., 1951; MILLER and NOSSAL, 1964; WHITE et al., 1967), on autoradiography of isotopically labeled antigens (CHENG et al., 1961; ROBERTS and HAUROWITZ, 1962; ADA et al., 1964; NOSSAL et al., 1964, 1965b, 1966, 1968a; MCDEVITT et al., 1966; LANG and ADA, 1967; HANNA et al., 1967; HANNA and SZAKAL, 1968), and on electron-dense antigens (WELLENSIEK and COONS, 1964). These investigations revealed that when marker protein antigens were injected intravenously they were diffusely distributed throughout the marginal zones and the red pulp by 2 hours, with practically no antigen being detected in the white pulp. Within a day or two, however, the red pulp was essentially cleared of antigen; virtually all the antigen in the spleen was localized in the white pulp, especially in the region of the germinal center cap, but never in the area immediately surrounding the central arteriole. Antigen was retained in the germinal center for several weeks. Electron microscopic autoradiography revealed that antigens localized in the germinal centers were retained extracellularly in the complicated infoldings of dendritic reticular cells. These antigen-trapping cells were in close association with blast cells. LANG and ADA (1967) demonstrated that antigen-trapping by the dendritic reticular cells was dependent on the presence of antibody or antibody-like factors. These studies indicate that the initial interaction between the antigen and the antigen-reactive cells may have occurred in the periphery of the white pulp.

A much-debated question has been whether or not antibody-forming cells contain antigen (e.g., see HAUROWITZ, 1965). ROBERTS (1964) and WELLENSIEK and COONS (1964), using relatively large doses of radioactive protein antigens and electron-dense, metal-containing protein antigens, respectively, demonstrated that blast cells and many immature plasma cells contained antigen or fragments of antigen; mature plasma cells contained little or no antigen. On the other hand, NOSSAL et al. (1965a), employing very small doses of antigen, found practically no detectable antigen in singly isolated antibody-forming cells during the 7S phase of the primary response. In subsequent experiments in which earlier phases of the primary and secondary response were examined, it was indeed observed that significant numbers of immature antibody-forming cells (plasmablasts and immature plasma cells) contained detectable amounts of antigen (NOSSAL et al., 1967). The frequency of these antigen-containing cells was apparently dependent on the dose of antigen injected. It was emphasized, however, that many antibody-forming

cells contained little or no antigen. Thus it appears that antigen or antigen fragments *can* enter immature antibody-forming cells or their precursors, but the question still remains as to whether antigen is *required* to enter these cells in order to induce plasmacytopoiesis and antibody formation.

E. Antibody-Forming Cells

Splenic distribution studies have revealed that most of the antibody-forming cells are found in the red pulp, but infrequently a few can be seen in the germinal centers of the white pulp (COONS et al., 1955; LEDUC et al., 1955; ORTEGA and MELLORS, 1957; VAZQUEZ, 1961; WHITE et al., 1967). Morphologically, antibody-forming cells constitute a heterogeneous population. Most are plasma cells (LEDUC et al., 1955; VAZQUEZ, 1961; BANEY et al., 1962; DE PETRIS et al., 1963; URSO and MAKINODAN, 1963; BALFOUR et al., 1965), some are distinctly lymphocytes (VAZQUEZ, 1961; HARRIS et al., 1966; HUMMELER et al., 1966), and a few possess macrophage-like properties (BUSSARD and LURIE, 1967). As expected, there is morphological and functional variation even within a class of cells. In terms of antibodies synthesized, most, if not all, individual plasma cells produce immunoglobulins of a single specificity, class, and allotype (MELLORS and KORNGOLD, 1963; CHIAPPINO and PERNIS, 1964; PERNIS et al., 1965; CEBRA et al., 1966; GREEN et al., 1967; NUSSENZWEIG et al., 1968). There are all gradations of plasma cells, from immature to mature. Immature plasma cells possess the capacity to synthesize DNA and undergo mitosis, while mature plasma cells lack the capacity to proliferate (SCHOOLEY, 1961; MÄKELÄ and NOSSAL, 1962; URSO and MAKINODAN, 1963; BALFOUR et al., 1965; HARRIS, 1968).

Although it has been reported that some mature antibody-forming cells in the spleen enter the circulation (HULLINGER and SORKIN, 1963; LANDY et al., 1964; KEARNEY and HALLIDAY, 1965; CANNON and WISSLER, 1967; CHAPERON et al., 1968; HIRAOMTO et al., 1968), the ultimate fate of these cells is not fully understood. They can either colonize some other lymphoid organ or return to the spleen. However, eventually they must either die or dedifferentiate into nonfunctional cells. If mature antibody-forming cells dedifferentiate (WISSLER et al., 1957; LAVIA et al., 1960), they may transform into small lymphocytes, the most likely candidate for the "memory" cell (GOWANS, 1966; GOWANS and UHR, 1966; BOSMAN and FELDMAN, 1968). If the ultimate fate of antigen-triggered functional cells is death, then the path of differentiation of immunocompetent cells is unidirectional and suicidal (ALBRIGHT and MAKINODAN, 1965; MAKINODAN and ALBRIGHT, 1967). Evidence for the aging of antibody-forming cells is mainly derived from morphological studies: cells with heavily vacuolated cytoplasm (MAKINODAN et al., 1954; FELDMAN, 1964) and abnormal nuclei (multilobulated nuclei, fragmented nuclei, nuclei with bridges, etc.) (SADO et al., 1964; and unpublished) have been observed, and their frequency seems to be increased at the end of the log phase of the response (MAKINODAN et al., 1954; SADO et al., 1964; and unpublished). An additional support for this view, that death is the ultimate fate of these cells,

comes from the observation that the number of plasma cells phagocytized in the spleen is most frequent at the end of the log phase (SWARTZENDRUBER, 1964).

F. Summary

These studies have shown that there are three distinct sites of proliferation in the spleen following antigenic stimulation. Each site is a complex structure made up of reticuloendothelial cells and lymphoid cells of thymus and bone marrow origin which are apparently turning over constantly. The first site of proliferation essential for the production of antibody-forming cells occurs in the white pulp surrounding the the central arterioles 12 hours after antigenic stimulation. This proliferation appears to be related to the production of 19 S (IgM) antibody-forming cells. Later, proliferation commences simultaneously at the second and third sites and is related to the production of 7 S (IgG and IgA) antibody-forming cells. One of these sites is the germinal center, where antigens are selectively trapped by dendritic reticular cells. The other is the red pulp, where most of the antibody-forming cells are found.

III. Cellular Events in Initiation of Antibody Formation

A. Scope

This section will deal with the cellular aspects of antigen-induced initiation of antibody formation. For the purpose of this review, the term "initiation" will be defined as the antigen-induced events leading to the *initial* production of antibody-forming cells. Although antigen and its manipulation during initiation are of decisive importance in the production of specific antibody-forming cells, neither the role of antigen nor the molecular aspects of initiation will be considered here. The discussion will be limited to a consideration of the cells, and the interactions between them, that are crucial to the initiation of antibody formation.

As will be shown below, it seems clear at the present time that the generation of antibody-forming cells most probably involves an interaction among cell types. Thus, the ensuing discussion will be divided into two parts: 1. a consideration of the evidence for the interaction among cell types during the initiation process, and 2. a consideration of possible modes of interaction between the cells involved.

B. Evidence for Interaction Among Cell Types

The concept of interaction between cells in the initiation of the immune response is not particularly new, but our current interest probably stems from the early work of FISHMAN (1959, 1961). He demonstrated that a cell-free homogenate, prepared from rat peritoneal cells previously incubated with T2 bacteriophage as antigen, would stimulate the synthesis of specific antibody when added to cultures of normal rat lymph node cells. Since lymph node cells plus antigen, or peritoneal cells plus antigen, failed to produce antibody, it was concluded that cell types from both populations were required. And

further, the data suggested that the mechanism of initiation was sequential, in that, the peritoneal cells, rich in macrophages, had to first prepare a ribonuclease-sensitive material, which in turn would induce lymph node cells to synthesize antibody. While these investigations have been extended in various directions (FISHMAN and ADLER, 1963; FISHMAN et al., 1965; ADLER et al., 1966; FISHMAN and ADLER, 1968), the point to be made here is that the interaction of two populations of cells was required for the induction of antibody synthesis. Other investigators, employing rabbits (PRIBNOW and SILVERMAN, 1967) and mice (GALLILY and FELDMAN, 1967; FELDMAN and GALLILY, 1968) and using other antigens, have also obtained evidence that although lymphoid cells synthesize antibody, they cannot be induced to do so without the aid of another cell type. Most evidence, though circumstantial, suggests that a macrophage-like cell may be the auxiliary cell.

Requirement for the interaction of two cell populations *in vitro* has been demonstrated by MOSIER (1967), who used the culture technique of MISHELL and DUTTON (1967). This culture system supports the *in vitro* initiation of a primary hemolysin response to foreign erythrocytes by spleen cells. MOSIER found that mouse spleen cells could be separated into two populations on the basis of their ability or inability to adhere to the bottom of plastic petri dishes. A response to sheep erythrocytes occurred only when the two populations, adherent and nonadherent cells, were mixed.

From the work of those investigators cited above, it is difficult to escape the conclusion that initiation of the immune response does indeed involve an interaction between cell types. There is another important body of information which contributes to the idea that interaction is required for initiation; these studies have been concerned mainly with determining the source of immunocompetent cells and stem from the now well known observation that the thymus plays a decisive role in developing and maintaining the individual's immunological system. A very selective presentation of this material will now be given, with exclusive reference to the problem of interaction in the generation of antibody-forming cells.

CLAMAN et al. (1966), in experiments designed to test for the presence of potentially immunocompetent cells in the thymus, discovered a synergistic effect between thymus and bone marrow cells. They followed the production of hemolytic foci in the spleens of irradiated mice reconstituted with thymus cells, bone marrow cells, and mixtures of thymus and bone marrow cells. The activity of the mixed population was greater than could be accounted for by summation of the separate activities of the thymus and bone marrow populations. They were not able, however, to determine which of the two populations provided precursors of antibody-forming cells and which acted in an auxiliary fashion.

The report of DAVIES et al. (1967) was one of the first indicating that thymus-derived cells were probably not the precursors of antibody-forming cells. They studied the secondary response to sheep erythrocytes in lethally irradiated CBA and CBA/T6T6 mice, previously rendered isoimmune to cells

of the other strain. These mice were reconstituted with primed spleen cells from chimeric donors containing CBA bone marrow-derived cells and CBA/T6T6 thymus-derived cells. On challenge with sheep erythrocytes, only recipients isoimmune to CBA/T6T6 cells supported a significant immune response, indicating that the thymus-derived cells of the chimeric spleens were *not* synthesizing antibody. Recipients isoimmune to CBA cells, however, failed to make a significant response. This result demonstrated that the CBA bone marrow-derived cells of the donor spleens were most probably the precursors of the antibody-forming cells. A similar conclusion had been reached previously by others (FORD et al., 1957; TRENTIN and FAHLBERG, 1963). Since the control recipients, non-isoimmune mice of both strains, exhibited relatively high responses, there was an indication of a synergistic effect or interaction between thymus-derived and bone marrow-derived cells.

MILLER and MITCHELL and their collaborators have recently published a series of papers encompassing their studies relevant to the interaction of cell types in the generation of antibody-forming cells (MILLER and MITCHELL, 1968; MITCHELL and MILLER, 1968b; NOSSAL et al., 1968b; MARTIN and MILLER, 1968). It was shown that thymus or thoracic duct lymphocytes could restore the ability of neonatally thymectomized mice to muster a primary immune response to sheep erythrocytes. Through the use of neonatally thymectomized mice reconstituted with allogeneic or F_1 thymus or thoracic duct lymphocytes, and employing anti-H2 sera directed against the donor cells, it was found that hemolysin-producing plaque-forming cells were of host origin. Thus, the thymus or thoracic duct cells were not providing the precursors of the antibody-forming cells detected in the recipient mice.

Using young adult recipient mice that had been thymectomized and irradiated, it was demonstrated that thoracic duct lymphocytes or a combination of thymus and bone marrow cells would restore competence. Thymus or bone marrow cells alone would not. Through the use of anti-H2 sera, hemolysin-producing cells were shown to be of bone marrow origin. The conclusion that the bone marrow constitutes the source of precursors of antibody-forming cells was confirmed by the analysis of marker chromosomes in single antibody-forming cells using the CBA-CBA/T6T6 system.

From these investigations and others (MILLER and MITCHELL, 1967; MITCHELL and MILLER, 1968a), MILLER and MITCHELL and co-workers have made the following conclusions. Two cell types are required for the initiation of the immune response to sheep erythrocytes. One is derived from bone marrow and is the precursor of antibody-forming cells. The other, derived from the thymus, represents the cell initially sensitive to antigen, and has been termed the antigen-reactive cell. Antigen-reactive cells interact with precursor cells and in some manner influence the differentiation of the latter into antibody-forming cells.

The statements of the preceding paragraph constitute a synopsis of our current understanding of the source of cells involved in the initiation of the immune response. Coupled with the work of others mentioned above, it seems

that the *initial, effective* contact with antigen is made by thymus-derived, antigen-reactive cells. The antigen-reactive cell presumably manipulates the antigen and passes on specific information to bone marrow-derived precursor cells which give rise to a population of antibody-forming cells through division. The information transferred during the interaction of antigen-reactive cells and precursor cells may be in the form of modified antigen molecules, fragments of antigen molecules (possibly determinant groups coupled to RNA), or informational analogues of antigen determinants. Whatever the mechanism of this information transfer, it is clear that antigen-reactive cells and precursor cells must interact. Hypotheses concerning the possible form of this interaction will be the final consideration of this section.

C. Possible Modes of Cellular Interaction

It has been mentioned previously that MOSIER was able to separate mouse spleen cells into two populations, adherent and nonadherent cells, neither of which, alone, would respond to antigen by producing antibody-forming cells *in vitro* (MOSIER, 1967). Combining the two populations resulted in the generation of hemolysin-producing cells in tissue culture upon stimulation with sheep erythrocytes. In a recent publication, MOSIER and COPPLESON (1968) have attempted to decipher the mechanism of the interaction between these cells by conducting dose-response experiments for one cell type (population) in an excess of the other type. It was concluded that the basic immunologically competent unit consisted of three cells — one adherent cell and two nonadherent cells. If the two nonadherent cells were different, three cell types interacted. If they were the same, only two cell types were interacting. Since adherent cells could be pretreated with antigen and a normal response obtained by adding nonadherent cells, it seems that the adherent population contains the antigen-reactive cell. The nonadherent population most likely contains the precursor cells. Important to material presented below is the point that, here, the basic immunologically competent unit exists in a single active configuration — three cells. A single unit of response is given by the interaction of an adherent cell (antigen-reactive cell) with two, and only two, nonadherent cells, at least one of which must be a precursor cell. Thus, in a dose-response curve, the increase in number of antibody-forming cells with increasing spleen cell dose is due to the increase in number of these three-cell complexes. MOSIER and COPPLESON found the slope of a logarithmic dose-response curve to be about 2.6. The model suggested by their data dictates that the increase in the number of three-cell complexes with increasing spleen cell dose follow this same slope. As will be seen, these facts result in a prediction which contrasts with a similar one based on the second interaction model to be presented.

GROVES et al. (1969) have proposed a significantly different model for the interaction of antigen-reactive cells and precursor cells. The model is stochastic in nature and is based on interpretation of two types of experimental data, namely, limiting dilution analysis and dose-response data. In this model the

antigen-reactive cell is assumed to have multiple interaction sites and is thus able to interact with more than one or two precursor cells. The actual number of precursor cells which interact with an individual antigen-reactive cell is a function of the lymphoid cell dose. Thus, there are several configura-

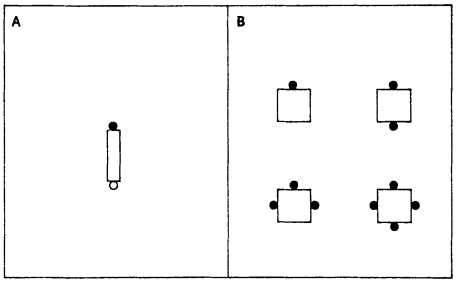


Fig. 2A and B. Comparison of possible configurations for immunologically competent interaction units in the model of MOSIER and COPPLESON (1968) (A) and that of GROVES et al. (1969) (B). Rectangle or squares represent antigen-reactive cells, closed circles represent precursor cells, the open circle may or may not be a precursor cell (see text). In "B" the antigen-reactive cells are depicted as having four interaction sites

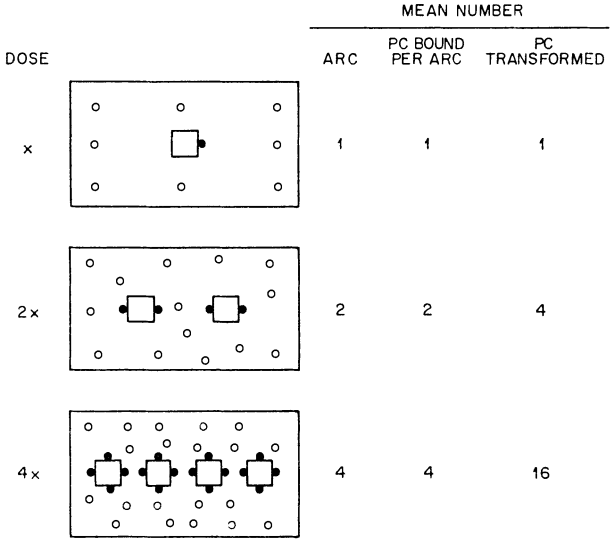


Fig. 3. A diagramatic representation of the interaction model of GROVES et al. (1969). Squares represent antigen-reactive cells, open circles represent unbound precursor cells, and closed circles represent bound (or transformed) precursor cells. See text for details

tions for the basic immunologically competent interaction units. The possible configurations of immunologically competent interaction units for the model of MOSIER and COPPLESON and this model are contrasted in Fig. 2.

The model of GROVES et al. was constructed to explain a biphasic logarithmic dose-response curve which has been observed in both cell transfer and diffusion chamber experiments (BOSMA et al., 1968; GROVES, 1968). At low spleen cell doses a slope of 2 was observed, but at higher doses there was an

abrupt transition to a slope of 1. For the purpose of this review, only the portion exhibiting a slope of 2 at low spleen cell doses need be considered.

The basic concept of the model should be clarified by the qualitative description presented in Fig. 3. An antigen-reactive cell interacts with precursor cells, which become transformed into antibody-forming cells. Although not depicted in the figure, these newly formed antibody-forming cells are assumed to undergo a fixed number of divisions. At unit dose of lymphoid cells, X, there is a mean of one antigen-reactive cell per sample. In this hypothetical example, the antigen-reactive cell has four interaction sites. At dose X, there is a mean of one precursor cell bound per antigen-reactive cell. This bound precursor cell becomes transformed into an antibody-forming cell which divides y times, yielding a total of 2^y antibody-forming cells. When the dose is doubled, to $2X$, there is a mean of two antigen-reactive cells per sample; since the mean number of precursor cells bound per antigen-reactive cell is a linear function of the dose, these antigen-reactive cells would be expected to bind two precursor cells each. Thus a total of four precursor cells, on the average, become transformed into antibody-forming cells at a dose of $2X$. Each of these dividing y times produces a total of (4) 2^y-antibody-forming cells. This same reasoning applies through a dose of $4X$. It will be noted that the mean number of precursor cells transformed and the total number of antibody-forming cells produced have followed a relationship with the dose having a slope of 2 in a logarithmic plot. That is, for every doubling of the dose, there is a fourfold increase in the total number of precursor cells bound or the number of antibody-forming cells produced.

A major difference between this model and that of MOSIER and COPPLESON will be noted by an examination of Fig. 3. While the number of precursor cells transformed increases with a slope of 2 in relation to the dose, the number of immunologically competent interaction units increases with a slope of 1. It will be recalled that the model of MOSIER and COPPLESON requires that the number of interaction units increase with the same slope as that for the number of precursor cells transformed. These differences result in conflicting predictions concerning the concomitant relationship between the number of hemolytic foci per spleen and the maximum number of antibody-forming cells produced per spleen as functions of spleen cell dose. On the basis of both models, the site of a hemolytic focus in the spleen of an irradiated recipient would be determined by the position at which an antigen-reactive cell lodges and interacts with precursor cells. Thus, MOSIER and COPPLESON'S model predicts that both the number of foci per spleen and the number of antibody-forming cells per spleen would increase with the same slope as the dose of spleen cells increased; that is, with the slope of 2.6 observed in their logarithmic dose-response curve. The model of GROVES et al., on the other hand, predicts that the number of foci per spleen would increase with a slope of 1, while the number of antibody-forming cells per spleen increases with a slope of 2. These latter predictions have been confirmed by the experiments of GREGORY and LAJTHA (1968). The features of this model which account for

these predictions are that an individual antigen-reactive cell is able to interact with and cause the transformation of multiple precursor cells, and that the number of precursor cells transformed per antigen-reactive cell is a linear function of lymphoid cell dose.

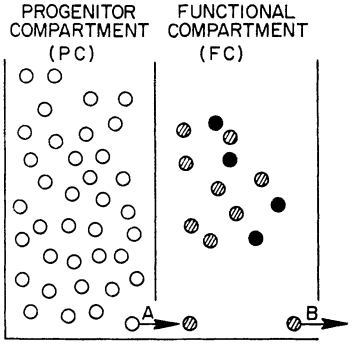


Fig. 4. Cellular differentiation in a two-compartment system. ○ Progenitor cell. ◍ Antibody-forming cell, dividing. ● Antibody-forming cell, nondividing. A Recruitment. B Emigration and death (physical and/or functional). Generation time (GT): time required for a proliferating cell to complete one cell cycle. Doubling time (DT): time required for the functional compartment to double in size.

$$DT = f\left(\frac{dA}{dt} - \frac{dB}{dt}; GT\right)$$

D. Summary

The evidence indicates clearly that at least two cell types are required for the initiation events leading to the generation of antibody-forming cells. These are the thymus-derived antigen-reactive cells and the bone marrow-derived precursor cells. The actual mechanism for the interaction of these two cell types remains to be determined, but we have discussed two models which describe possible modes for this interaction.

IV. Growth Models

A. General Considerations and Assumptions

In the following sections, we shall attempt a systematic presentation of growth patterns for populations of antibody-forming cells. A discussion of some of the available experimental data relevant to growth patterns will also be included.

In the generation of these growth patterns, the response is considered to arise from the transformation of undifferentiated progenitor cells[7] into functional antibody-forming cells. That is, new antibody-forming cells are not considered to arise by proliferation of background cells. This assumption is consistent with the work of HEGE and COLE (1967), in which they showed that the background activity did not proliferate; rather, the background anti-

[7] "Progenitor cells" are defined as the *immediate* precursors of antibody-forming cells.

body-forming cells were shown to be essentially a nonproliferating population of "long-lived" cells.

Models for the antigen-triggered increase in the number of antibody-forming cells in the primary response will be categorized in terms of predicted types of recruitment. "Recruitment" will be defined as the enlistment of cells from the progenitor compartment into the functional compartment (see Fig. 4). "Single recruitment" will be limited to a single recruitment event, of arbitrary magnitude, occurring at a given time. Multiple recruitment may be of two types. "Nonrandom multiple recruitment" will refer to numerous recruitment events spaced periodically, usually as some constant function of the generation time. "Random multiple recruitment" will refer to numerous recruitment events randomly spaced in time.

The cells which are recruited may differ in their proliferating capacities (dividing or nondividing). "Synchronous division" refers to "in phase" division of the entire proliferating population. "Asynchronous division" refers to "out of phase" division of the proliferating population. Whether the progenitor population is proliferating prior to recruitment is not considered because it has no demonstrable effect upon the models or growth patterns as discussed in this review.

The graphical presentations will be given in terms of the number of antibody-forming cells, in arbitrary units, and time. Time will be expressed in arbitrary units or in numbers of generations. These growth patterns are presented as semilogarithmic graphs with the number of antibody-forming cells plotted on the logarithmic scale. The term "linear growth patterns" will be used for exponential functions yielding straight lines in semilogarithmic plots; "staircase growth patterns" are discontinuous exponential functions, the second general type of pattern. The illustrations below some of these growth patterns are secondary to the patterns themselves and should be considered only as visual aids.

The growth patterns considered will be limited by the requirement that they conform to the exponential portion of experimentally observed patterns (see Fig. 5). This requires that the patterns increase in an exponential and essentially monophasic manner. It should be noted that the staircase patterns to be considered do not violate this condition. It is entirely possible and indeed probable that kinetic assessments of the growth of the primary immune response have lacked sufficient detail; i.e., truly nonlinear patterns approach linearity as the assessment interval increases. Kinetic evidence is therefore currently lacking in most systems as support for or against any of the growth patterns to be presented here.

"Generation time", the time required for a proliferating cell to complete one cell cycle ($G_1 + S + G_2 + M$), is assumed to be constant. "Doubling time" will be defined as the time required for the functional compartment, containing the antibody-forming cells, to double its size. Doubling time must therefore be a function of the rate of recruitment, dA/dt, and the rate of emigration and death, dB/dt, in addition to the generation time (see Fig. 4). The diffe-

rence between doubling time and generation time have been discussed in detail by TANNENBERG and MALAVIYA (1968). The term "death" will be used in the functional sense (i.e., loss of antibody-forming activity) as well as in the physical sense. In the building of the models, death will not be a primary consideration.

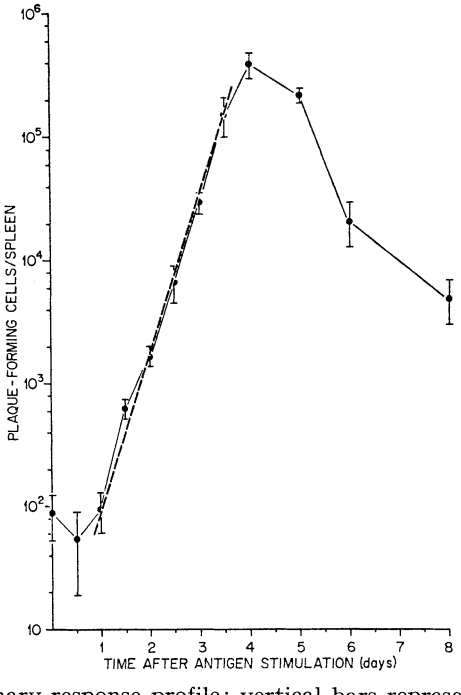


Fig. 5. "Typical" primary response profile; vertical bars represent two standard errors

Recruitment of progenitor cells into the functional compartment will be restricted to those in the G_1 period of the cell cycle (QUASTLER and SHERMAN, 1959; QUASTLER, 1963). This will thus limit the degree of asynchrony in the recruited population. Assuming that G_1 comprises one quarter, at most, of the total cell cycle (SADO and MAKINODAN, 1964), no cell may divide more than one quarter of a cell cycle out of phase.

Only models for the extreme cases will be considered; that is, single recruitment or multiple recruitment (nonrandom and random) of nondividing, synchronously dividing, or asynchronously dividing antibody-forming cells. Mixed and hybrid models will be left for interested and more ambitious individuals. Antigen dose, which is also a contributing variable to the overall primary response, will not be considered in the models.

B. Single-Recruitment Models

In a single-recruitment model with no proliferation, the magnitude of the response is limited by the magnitude of the recruitment (see Fig. 6). The kinetics of such a response require essentially instantaneous attainment of peak activity. The rate of decline of the response must be dependent on the

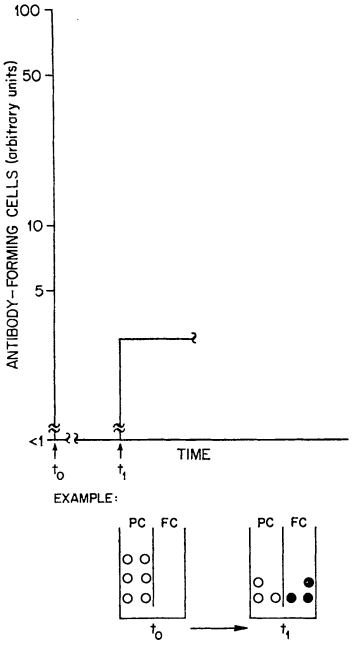


Fig. 6. Single recruitment, no division

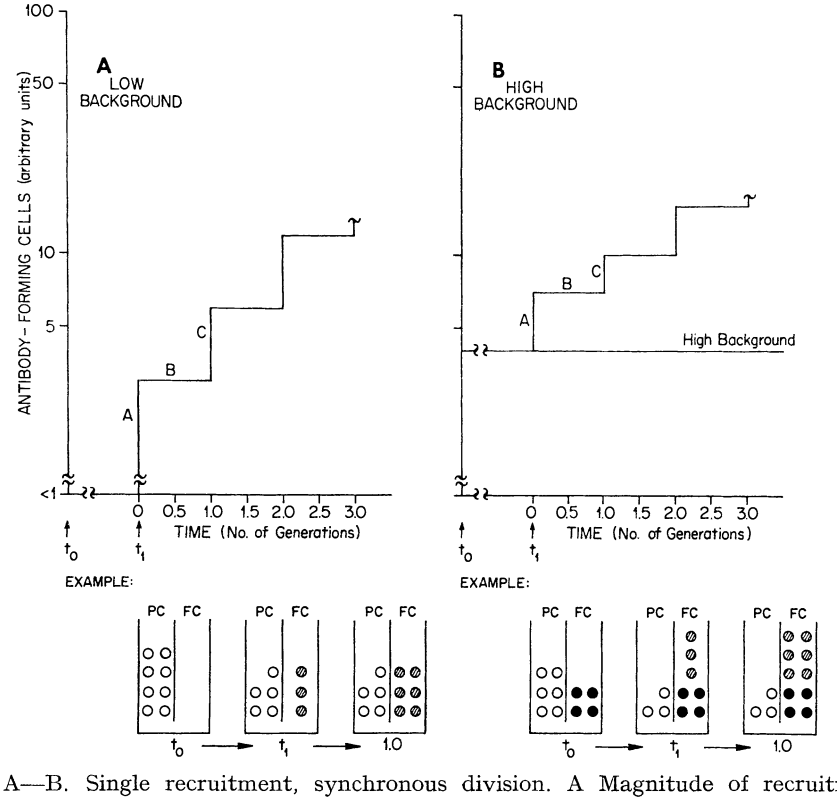


Fig. 7 A—B. Single recruitment, synchronous division. A Magnitude of recruitment. B Shelf-time. C Amplification

rate of emigration and death. Seemingly, a single-recruitment event with no proliferation of the functional antibody-forming cells fails to adequately describe the *in vivo* situation. Models such as this, however, may be applicable to certain *in vitro* systems.

Single recruitment with synchronous division yields a staircase pattern (see Fig. 7A). The ultimate magnitude of the response is dependent on the number of cells recruited and the number of divisions allowed. The initial

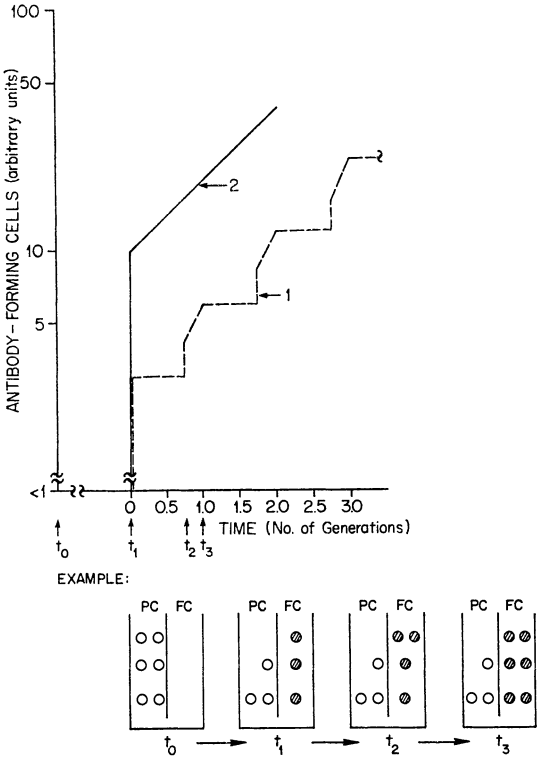


Fig. 8. Single recruitment, asynchronous division. 1 restricted asynchrony; 2 unrestricted asynchrony

increase in antibody-forming activity, A, is representative of the number of cells recruited. The "shelf-time", B, is equivalent to the generation time of the proliferating antibody-forming cells. Provided that background is low in comparison with the magnitude of recruitment, the amplitude of increase in activity, C, is always twofold. If background is high, the amplitude will represent less than a twofold increase in total activity, until the contribution of the background becomes negligible (see Fig. 7B). Experimental evidence for synchronous division of an antibody-forming cell population recruited as a single event is currently lacking.

Asynchronous division of the recruited population represents the final alternative for single-recruitment models. The growth patterns idealized in Fig. 8 will, of course, depend not only on the magnitude of the recruitment, but on the degree of asynchrony of the dividing population. This asynchronous

division is illustrated in the figure. Curve 1 depicts the result where strict adherence to a constant generation time, with differentiation occurring during the G_1 period, is maintained. The G_1 period was assumed to comprise one quarter of the cell cycle. All cells were recruited at the same time, but were randomly distributed throughout the G_1 period. Curve 2 may be generated when recruitment takes place at any portion of the cell cycle and the magnitude of the recruitment is large.

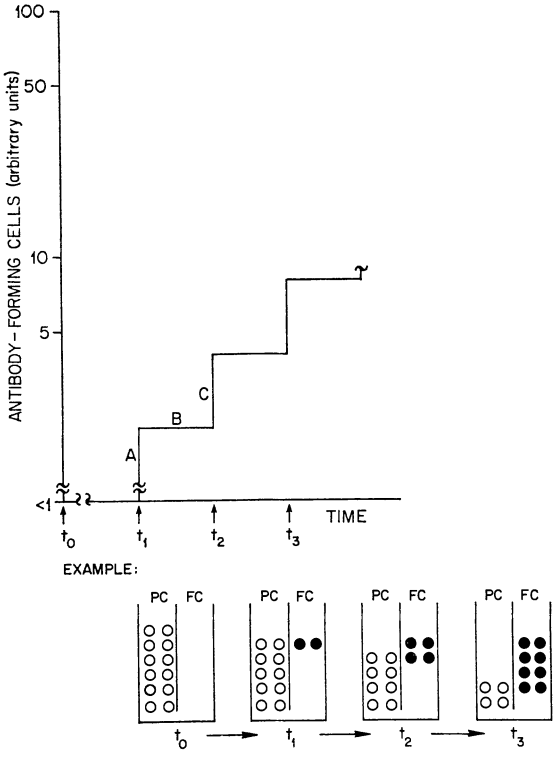


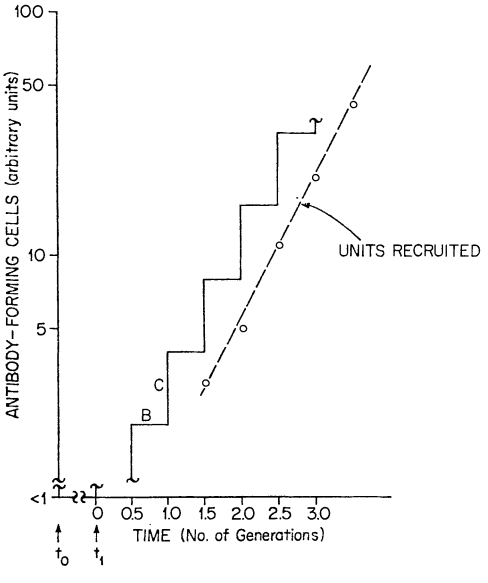
Fig. 9. Nonrandom multiple recruitment, no division. A Magnitude of recruitment. B Shelf-time. C Amplification

C. Nonrandom Multiple-Recruitment Models

Recruitment occurring in a nonrandom manner without proliferation of antibody-forming cells can generate a "typical" response curve only under conditions of exponentially increasing recruitment (see Fig. 9). The initial increase in activity over background is, as always, representative of the number of cells recruited from the progenitor population. However, the shelf-time, B, now represents only the interval between recruitment events and is not a function of the generation time. The amplitude, C, represents only the magnitude of the second recruitment. Amplification, however, must not vary significantly about some mean value if a linear monophasic response is to be maintained. The degree of amplification required will be entirely dependent upon the interval between recruitment events, B. Shorter intervals would require fewer cells to be recruited initially, thus resulting in a smaller ampli-

fication. This model or a modification of it may be applicable to certain *in vitro* systems.

Synchronous division of cells recruited in nonrandom multiple events yields another staircase pattern (see Fig. 10). The pattern is seen experimentally only for cases where the interval of assessment of antibody-forming activity is much shorter than the shelf-time or interval between recruitments, B. This pattern represents the special case for which recruitment occurs at intervals of one-half the cell cycle. The shelf-time, B, is only equivalent to the



TIME	0	0.5	1.0	1.5	2.0	2.5	3.0	3.5	4.0
RECRUITMENT and PROLIFERATION	1	1	2	2	4	4	8	8	16
		1	1	2	2	4	4	8	8
			1	1	2	2	4	4	8
				3	3	6	6	12	12
					5	5	10	10	20
						11	11	22	22
							21	21	42
								43	43
									85
TOTAL in FC	1	2	4	8	16	32	64	128	256

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Fig. 10. Nonrandom multiple recruitment, synchronous division. B Shelf-time. C Amplification. The numbers below the diagonal line represent the number of units recruited to maintain an exponential response. The numbers above the diagonal line represent the division of the recruited units

generation time when the interval between recruitments is equal to the generation time; the amplification for this special case is, however, twofold. To maintain a monophasic response profile, amplification will generally remain constant for all cases. The shelf-time represents the doubling time when background contribution to total activity is negligible and amplification is twofold. If the total response is to be exponential, recruitment must always increase in a virtually exponential manner as shown in Fig. 10.

An asynchronously dividing population of cells recruited in multiple nonrandom events may approach a linear pattern (see Fig. 11). The maintenance of an exponential response again requires that recruitment increase

exponentially. The pattern's linearity, however, is a function of the size and asynchrony of the proliferating antibody-forming cell population, analogous to single recruitment of asynchronously dividing cells. The interval between recruitments will also contribute to the multiplicity of patterns possible for this type of model. For example, short intervals between recruitment events of large numbers of cells yields a more linear pattern than long intervals between recruitment events. Typically, however, a sufficiently detailed pattern would show divergence from linearity at times of recruitment.

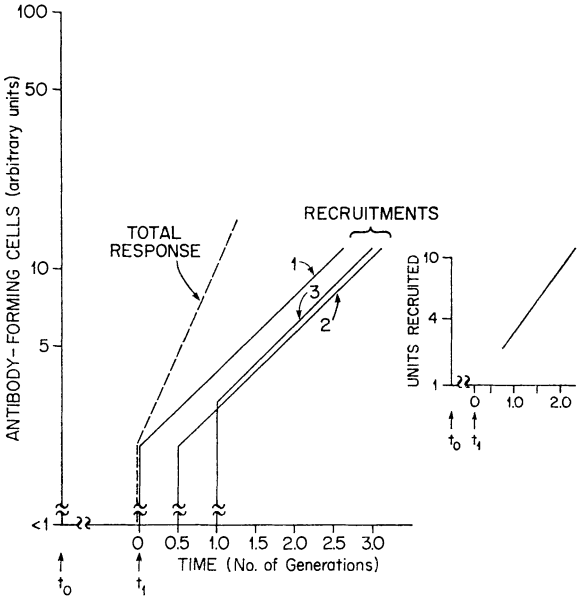

Fig. 11. Nonrandom multiple recruitment, asynchronous division. Individual recruitment events: 1, 2, and 3

D. Random Multiple-Recruitment Models

Random multiple recruitment with nonproliferation of antibody-forming cells is another complex situation; the true shape of the pattern is dependent upon the length of the interval between recruitment events (see Fig. 12). A combination of staircase and linear growth patterns is possible for cases where the interval between recruitments varies from longer (curve 1) to shorter (curve 2) periods of time. The pattern seen experimentally will, of course, depend upon the time interval between assessments of the antibody-forming activity. The longer the assessment interval, the more likely a linear pattern will result, as shown in Fig. 12, curve 2. When the background is high in comparison with the initial recruitment, the response may show a lag before it becomes exponential.

Random multiple recruitment of antibody-forming cells, dividing synchronously, represents another complex case (see Fig. 13). The pattern is strictly staircase with a shelf-time, B, varying as a function of the time interval between recruitment events. Amplification, C, varies in a complex manner, dependent upon the size of recruitment, the time of recruitment, and the

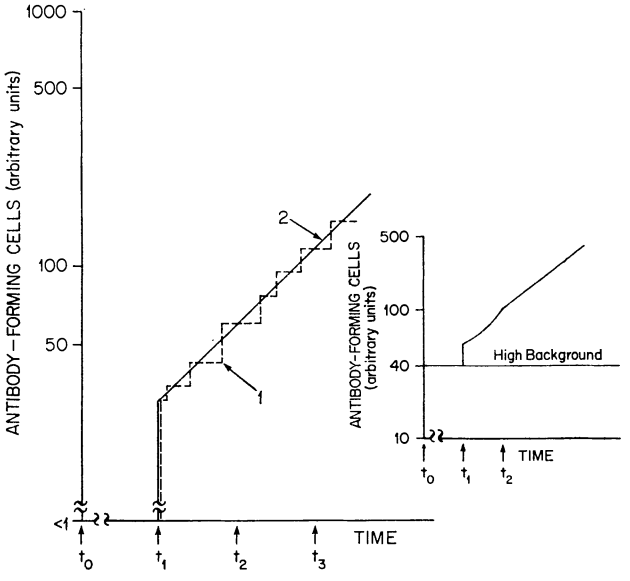

Fig. 12. Random multiple recruitment, no division

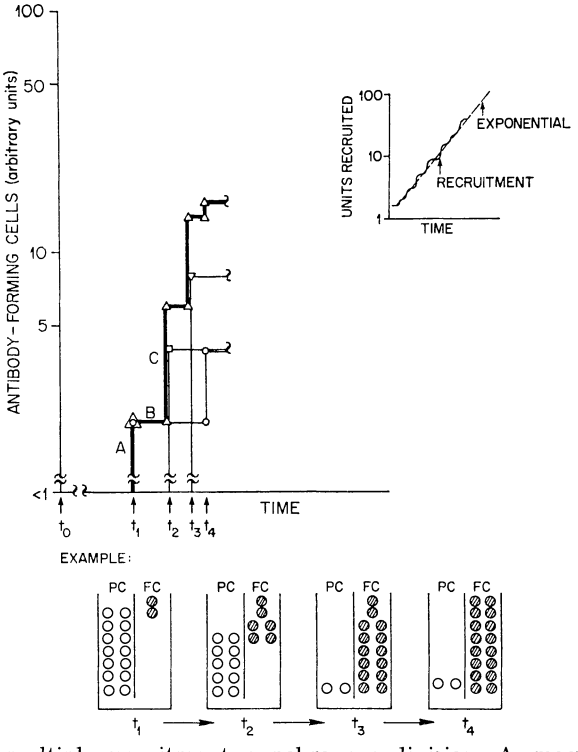

Fig. 13. Random multiple recruitment, synchronous division. A, magnitude of recruitment. B Shelf-time. C Amplification. Individual recruitment events: ○, ▽, and □. Total activity, △

generation time of the dividing population. For retention of a monophasic pattern, recruitment must vary around an exponentially increasing curve.

An asynchronously proliferating population recruited in a random multiple manner yields a growth pattern which will approach linearity depending on the kinetics of the recruitment events (see Fig. 14): as the recruitment events

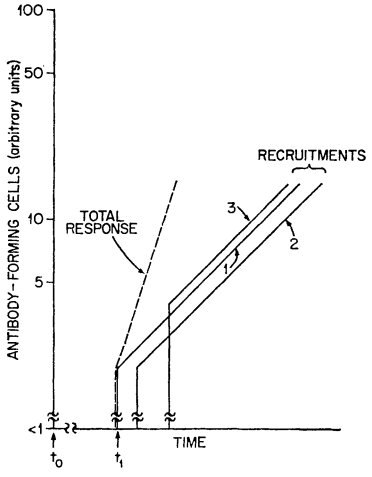

Fig. 14: Random multiple recruitment, asynchronous division. Individual recruitment events: 1, 2, and 3

occur more frequently and with increasing magnitude, the pattern approaches linearity. Previous discussion of asynchronous division and random multiple recruitment are applicable and shall not be repeated here (see Figs. 8, 11, 12, and 13). As long as recruitment continues, its magnitude must increase in an exponential manner. Failure to do so produces a nonlinear and/or multiphasic growth pattern.

E. Additional Considerations

In order that a comprehensive response profile be obtained, all of the proposed models require the operation of certain homeostatic control mechanisms. In general, there is a need for the quantitative control of recruitment, death, and emigration processes operating on both progenitor and functional compartments. The random-multiple recruitment models require especially sophisticated control mechanisms to prevent variable "overshoot" of recruitment (see Fig. 13); in control of this variable "overshoot", death may be a discontinuous or periodic process. Control must be in terms of both the time and magnitude of recruitment. Limitation on the extent of proliferation of a given cell may be in terms of its life-span or the maximum number of cell cycles. This distinction is critical for a comprehensive model.

It seems most likely that recruitment will terminate or be drastically reduced at the peak of the response, perhaps by depletion of the progenitor compartment, as proposed by DUTTON and MISHELL (1968). However, control mechanisms more sophisticated than this depletion may also explain the decline of antibody-forming activity.

The roles of proliferation, emigration, death, and dedifferentiation in the decay of the primary immune response are poorly understood (SADO, unpublished; HULLINGER and SORKIN, 1963; LANDY et al., 1964; KEARNEY and HALLIDAY, 1965; CANNON and WISSLER, 1967; CHAPERON et al., 1968). For example, it has been hypothesized that termination of the response may be due to a limitation in the number of divisions which antibody-forming cells may undergo, but no real evidence exists. The effect of death upon the overall response is unknown. It may serve as an important controlling factor by tempering the effect of recruitment.

There are numerous parameters which can be varied to generate a "typical" response. Investigation of these parameters is of fundamental importance for the creation of a comprehensive model to explain the events following antigenic stimulation.

F. Experimental Evidence

An extended discussion of the pertinence of our growth models to existing experimental evidence is not possible at the present time. We are aware of only one study in which the kinetic data are sufficient to discriminate between the various growth models. Nevertheless, we will consider selected experimental data dealing with the kinetics of the immune response.

There is considerable difference between *in vitro* and *in vivo* systems used in the study of the primary immune response. No single growth model should be expected to adequately explain all the systems. There is, however, great need to recognize the particular models representative of an individual system; this information is critical to the ultimate goal — applicability to a "natural system".

Multiple recruitment of nonproliferating antibody-forming cells, i.e., transformation without division, seemingly describes the *in vitro* system of BUSSARD and LURIE (1967). These investigators used a technique involving the incorporation of peritoneal cells into carboxymethyl-cellulose gum. The immunocompetent cells in this system do not proliferate, in that they are unaffected by mitotic blocking agents.

DUTTON and MISHELL (1968), using the hot-pulse technique with tritiated thymidine to eliminate dividing cells, demonstrated in an *in vitro* system the necessity for multiple recruitment. A model was devised to explain the observed 4-hour doubling time of the functional compartment containing cells with a generation time of 8 hours. This model requires exponentially increasing numbers of progenitor cells to differentiate into antibody-forming cells. The model presented by DUTTON and MISHELL is consistent with the nonrandom multiple-recruitment models presented in this review.

ROWLEY et al. (1968) and KOROS et al. (1968) claim that their experimental data are proof of a single-recruitment, asynchronous-division model. ROWLEY and coworkers used mitotic blocking agents to estimate the cell cycle times of the antibody-forming cells. The increase observed in the primary response was attributed entirely to the "exponential division" of antibody-forming

cells. The magnitude of the immune response was said to depend on the rate of division of the cells responding to antigenic stimulation. On the basis of ROWLEY'S mitotic blocking experiments, generation times essentially equivalent to the doubling times of the antibody-forming cell population were proposed. In all cases, however, the interpretation of experimental data was dependent upon the assumption of complete asynchrony in the proliferating population.

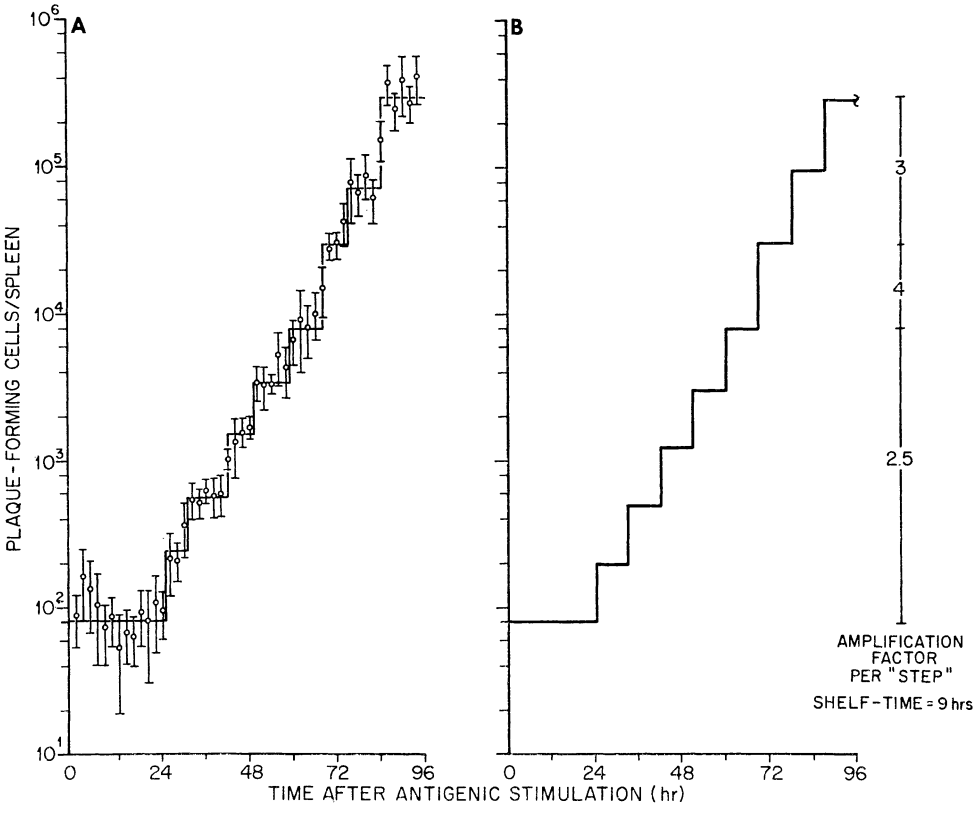

Fig. 15A and B. Experimental data of PERKINS et al. (1968) reflecting a model of nonrandom multiple recruitment, synchronous division. A Experimental data; vertical bars represent two standard errors. B Fitted model

PERKINS et al. (1968; and unpublished), however, have obtained evidence of significant *synchrony* for the early primary response in the spleen of the intact mouse (see Fig. 15 A). Their experimental data fit a nonrandom multiple-recruitment, synchronous-division model (see Fig. 15 B). A doubling time of about 6 hours was calculated from a regression line between 24 and 96 hours. The amplification was always greater than twofold, with a relatively constant shelf-time. The model therefore must be one in which recruitment is occurring nonrandomly, division is synchronous, and the interval between the recruitment events represents the generation. time. The shelf-time then gives a mean generation time of about 9 hours throughout the response.

G. Summary

Models for the growth of antibody-forming cell populations have been presented in a systematic manner. There is considerable need for reevaluation of the kinetics of the immune response, with assessment of antibody-forming activity at intervals much shorter than the doubling time. Sufficient data exist for only one *in situ* system. Further advancement of our knowledge concerning the growth of the immune response will require investigation of the homeostatic mechanisms controlling recruitment, emigration, death, and dedifferentiation.

V. Conclusion

In spite of the tremendous progress made in this area during the past decade, the cellular events responsible for the exponential rise in the number of antibody-forming cells following antigenic stimulation are still not understood. As the problem is too formidable for one to approach solely by studying the histology of tissues *in situ*, the primary method until recently, the lack of understanding is not unexpected. There have been, however, an increasing number of quantitative cellular studies performed *in situ*, and in *in vivo* and *in vitro* cultures. On the basis of these latter investigations we know that the primary cause for the increase in the number of antibody-forming cells in the spleen is due to a series of nonrandom recruitments of cells which subsequently proliferate. Furthermore, we are quite confident that at least two cell types are involved in initiating the antibody response.

It appears to us that more quantitative data in this area have been obtained during the past decade than during the previous 50 years. Therefore we are cautiously optimistic that this problem may be resolved within the next decade. To contribute to this objective, in this review we have evaluated current data in terms of model analysis, which we feel is the best approach at this stage of development.

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The Significance of the Dose of Antigen in Immunity and Tolerance

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With 8 Figures

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1. Introduction

The discovery of the phenomenon of immunological tolerance stimulated study of the significance of the size of the dose of antigen in immune phenomena. It has become apparent that antibody synthesis is not the only outcome of injecting antigen into an animal and that a specific inhibition of antibody synthesis can also occur. The size of antigen dose may determine whether or not immunity or tolerance is induced, but the physical nature of the antigen and the ontogenetic stage of development of the animal also play a significant part. Apart from the question of balance of the reaction between immunity

and tolerance, the significance of a study of the effect of the antigen dose as a factor which regulates the differentiation of immunologically competent cells acquired importance. The centre of these regulation processes appears to be the control of the molecular type of antibodies produced, which is reflected in the kinetics of the immune response and the development of immunological memory.

So far there has been no comprehensive review of the significance of antigen dose in various immune phenomena. However HAŠEK et al. (1961), SMITH (1961), MAKINODAN and ALBRIGHT (1967), ŠTERZL and SILVERSTEIN (1967) and UHR and FINKELSTEIN (1967) have sections which relate antigen dose to the subject of their reviews. For the sake of brevity detailed facts not actually related to the effect of antigen dose on the phenomena under consideration have been omitted.

The interest of immunologists in the theoretical evaluation of the effect of the antigen dose goes back many years. SMITH and ST. JOHN-BROOKS (1912) studied the dose — response relationship to typhoid bacilli in rabbits and found that the maximum agglutination and phagocytosis titres were a linear function of the antigen dose on a log — log plot. They pointed out that this log — log relationship was similar to the Freundlich adsorption isotherm equation and suggested that an antigen adsorption process was the limiting step in antibody production. STEVENS (1956) tested the equation against data from 13 published papers which gave a good fit within the given antigen dose limits. It appeared that as far as the maximum antibody response induced was concerned, an increase in the dose of protein antigen would be about 50 times more effective than the same increase for polysaccharide antigens, irrespective of the species of animal. Despite this simplification of the problem, we evidently still have no exact explanation for STEVEN'S basic claim, that "among the many reactions involved, the limiting step is an adsorption mechanism".

In discussing the molecular mechanisms of antibody synthesis we often find speculative comparison with the much better known mechanism of the induction of bacterial enzymes (JACOB and MONOD, 1961); the two systems have a number of features in common (MONOD, 1959). The unique aspect of antibody synthesis is the pronounced heterogeneity of the reactions in relation to changes in antigen dose, compared with the simple relationship between concentration of inducer and the amount of enzyme synthesis induced. It would therefore be of theoretical interest to evaluate how far polymorphism of the immune reaction is determined by the specific character of the actual induction process, or by the marked heterogeneity of the mammalian lymphoid cell population.

2. The Fate of Antigen

The relationship between dose of antigen and the immune response is dependent on the route by which the antigen is administered to the animal. Intravenous injection leads to instantaneous dilution of the antigen in the blood stream and tissues, while in local administration, especially in the form of a water-in oil emulsion, antigen persists for some time at the original con-

centration at the site of injection. Farr and Dixon (1960) found that a critical factor in the induction of antibody synthesis after local administration was the threshold of antigen concentration and not the total antigen dose. In experiments with protein antigens, they demonstrated in rabbits that a given dose was more effective as a more concentrated solution or alternatively that total antigen doses within a ten fold range of one another were equally effective if the concentration of antigen in the adjuvant was held constant. When comparing the antigen concentration in the aqueous phase of the adjuvant with that in the serum five minutes after intravenous administration, the time-course of the the dose-response curves was identical.

The way in which an antigen is eliminated after intravenous administration depends primarily on its physical nature. Some antigens such as serum proteins or ΦX bacteriophage, can persist for several days in the blood stream from which they are eliminated at a constant rate (half-life), while others disappear very quickly; for instance iodo- or azo-proteins, KLH, or T-2 phage, which have half-lives of only a few hours (Campbell and Garvey, 1963). Antigens which persist for some time extracellularly may react not only with "antigen-sensitive" cells present at the time of injection but also with cells which have differentiated from stem cells during the time of the persistence of antigen. Dose-response studies with non-circulating antigens such as KLH show a different pattern from that obtained with circulating antigens as in the latter case it is probably the outcome of the reaction of the antigen with only those cells present at the time of injection. The studies of Dixon et al. (1966, 1967) indicate that the size of the antibody response might be related to the amount of active immunogenic material present in the antigen, which may be only a portion of the total amount of antigen administered. As doses of KLH ranging from 1 to 1,000 mg stimulated very similar maximum antibody titres, it was suggested that the immunogen concentration actually active in stimulation of the immune response was relatively constant, irrespectively of the amount of antigen administered.

Detailed information on the mode of elimination of different doses of antigen is available for circulating antigens. It was found that, as the antigen dose was raised, the catabolic rate did not alter, but that the start of immune elimination was delayed by several days (Uhr et al., 1962; Iványi et al., 1964b). Experiments in chickens injected with ^{131}I-HSA (Iványi and Černý, 1965) enabled an evaluation to be made of the relationship between the dose of antigen injected and the level of antigen in the blood one day later and on the day of the onset of immune elimination (Fig. 1). Over a 10,000 fold range of antigen dose a linear increase occured in the concentration of antigen in the blood soon after immunization. Similarly, the amount of antigen in the blood increased at the time of the immune break in the elimination curve but reached a limiting value, which determines the "latent period" after massive doses of antigen. The threshold concentration of antigen sufficient for inhibition of the onset of immune elimination is evidently not the same as the dose which inhibits intracellular antibody synthesis. Despite of evidence, that large doses

of protein antigens led to delayed formation of antigen-antibody complexes in the blood stream and to a dalayed onset of cellular response in the spleen (ČERNÝ et al., 1965; see also section 5 b), it is clear that the early formation of serum antibody is significantly affected also by neutralization of some of the antibody by excess antigen (UHR et al., 1962; IVÁNYI et al., 1966). Differences in the antigen-binding properties of different types of antibody should be taken into account here. TERRES and SOREM (1965) demonstrated that the following generalization is true in vivo: [Ab] + [Ag] ⇌ [AgAb]. Furthermore

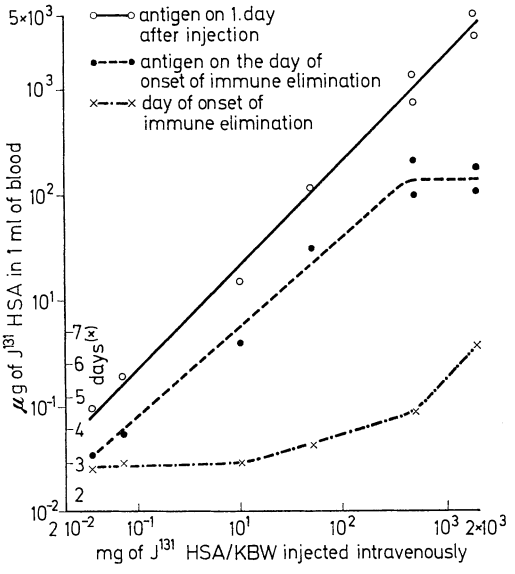

Fig. 1. The effect of antigen dose on its concentration in the blood of chickens immunized with various doses of I^{131} HSA. (From IVÁNYI and ČERNÝ, 1965)

they showed that the rate of elimination of antigen was a function of the ratio of free antibody to free antigen. The formation of a complex which is rapidly degraded requires an antibody/antigen ratio of over 5; however this does not always apply exactly because of the heterogeneity which exists amongst antibodies. In guinea pigs and rabbits, some antibodies appear to lead to the formation of complexes which are actually eliminated more slowly than the antigen itself (MORRISON and TERRES, 1965).

Several authors have found that medium sized doses of labelled antigens had approximately the same kinetics of elimination from spleen, liver and lungs. However using a very small dose of antigen, selective uptake of radioactivity in the spleen was demonstrated (Fig. 2). This can be explained by the presence of early 7S antibodies, demonstrated as an antigen/antibody complex with cytophilic properties to spleen cells in vitro (IVÁNYI, unpublished results). Similar results were obtained by FITCH et al. (1966), who found that selective localization of antigen in the lymphoid follicles of the spleen appeared at the same time as the onset of 7S antibody formation and this did not occur if there was only a 19 s response.

In many studies of distribution of antigen in different organs it is sometimes difficult to know whether its localization is directly relevant to the processes of the initiation of immunity or to the localization of macrophages involved in the degradation of the antigen-antibody complexes and/or excess free antigen. It would therefore be suitable to discuss here the question of the target cell with which antigen interacts after being injected into the animal. First of all there are the macrophages, which degrade antigen but whose

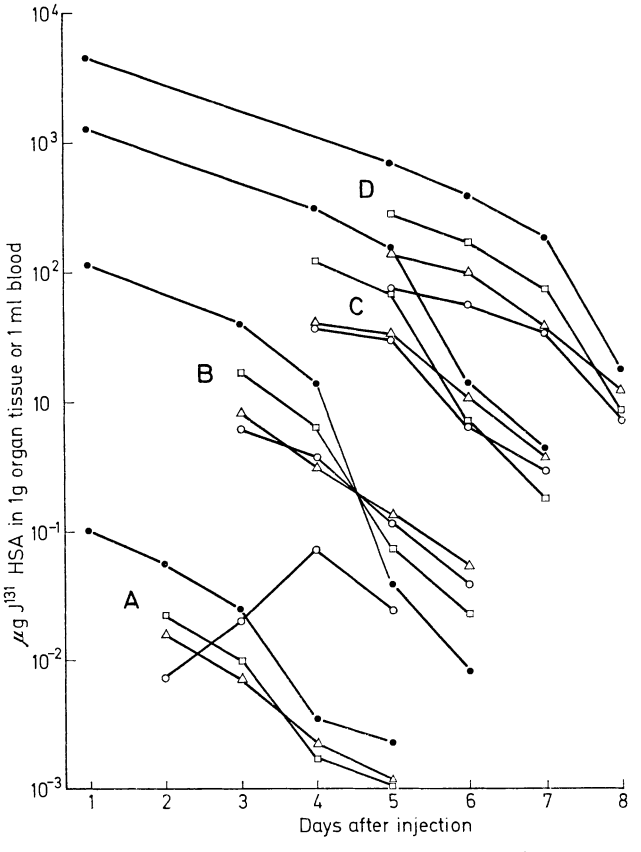

Fig. 2. Elimination curves of I^{131}HSA from blood and organs of chickens after injection of 0.35 (A), 50.0 (B), 500.0 (C), or 2,000.0 (D) mg HSA per kbw. ● blood, ○ spleen, △ liver, □ lungs. (From IVÁNYI and ČERNÝ, 1965)

significance in the induction of immunity is a subject of controversy. If we assume that the processing of antigen by macrophages is an integral part of immunization, we are offered the simple concept that the administration of antigen in a dose sufficient for saturation of the reticulo-endothelial system would lead to direct interaction of the antigen with the lymphoid cells and hence to the induction of tolerance (BRITTON and MOLLER, 1966). PERKINS and MAKINODAN (1965) however found that the incubation of peritoneal macrophages with antigen altered its immunogenicity, thus inhibiting an immune response after the addition of spleen cells. It could be assumed that in different

macrophages antigen is elaborated in very different ways (ROBERTS, 1964). However it has not yet proved possible to explain the quantitative relationships between antigen and different phagocyte populations experimentally (MAKINODAN and ALBRIGHT, 1967).

Recently interest has been evoked in the study of RNA preparations from immune macrophages in experiments where the injection of this material can lead to specific immunity. Some experiments of FISHMAN et al. (1965) demonstrated the significance of antigen dose in this respect. If macrophages are incubated with a low concentration of antigen, the antibody titre in recipients of RNA prepared from macrophages fed with antigen falls rapidly after the 4th day. With high antigen concentration the injection of RNA into normal animals produces an immune response with 2 maxima: the 1st on the 4th day and the 2nd on the 12th day, presumably representing the production of γM and γG antibodies.

3. Kinetics of Antibody Formation

a) Serological Studies

The rate of appearance and accumulation of antibodies in the serum of an animal have been discussed in several reviews (FREUND, 1953; BURNET and FENNER, 1949; INGRAHAM, 1964; UHR and FINKELSTEIN, 1967). Antibodies appear after a latent period and at first increase very rapidly, at an exponential rate and then more slowly at a linnear rate. Most of the total amount of antibody is synthesized in this last phase (TALIAFERRO, 1957; INGRAHAM, 1964). These different phases of the course of antibody production have given rise to concepts of the cellular and molecular background of antibody synthesis. The finding that this process was largely influenced by the dose of antigen was made in a number of experiments and raised new theoretical problems. In a series of experiments TALIAFERRO and TALIAFERRO (1957, 1963) simultaneously studied eight dose-response parameters of the formation of serum haemolysins against Forssman antigen in rabbits. On progressively raising the dose from 10^4 to 10^{10} SRBC, the peak titre of the response was increased and both the latent period and the duration of the exponential phase of serum antibody production was shortened, thus shortening the doubling time of the antibody titre. Assuming that serological data were also adequate criterion for the rate of cellular antibody synthesis, the authors interpreted the mechanism of the antigen dose/antibody response at the cell level primarily in terms of the shortening of the generation time of proliferating antibody-forming cells. These authors neither found signs of a delay in the onset of antibody formation nor a decrease in antibody titer even after maximum doses of antigen. Their results indicated, that the increase in the dose of SRBC resulted in: a) acceleration of diferentiation of antibody-producing cells; b) prolonged duration of antibody production.

Studies of total antibody titres do not take the existence of their different molecular and functional types into account. The regulatory role of the antigen dose in determining the molecular type of antibodies and the rate and amount

in which they are synthesized was discovered by UHR and FINKELSTEIN (1963) using bacteriophage ΦX and SVEHAG and MANDEL (1964a) using poliovirus. They found that with an adequately small immunization dose only γM antibodies are formed after primary immunization. After medium and large doses of antigen the production of γM antibody is succeeded by synthesis of γG antibodies. The threshold antigen dose for induction of the synthesis of γM antibodies is 100 times lower than that for γG antibodies. The duration of the latent period for γM antibodies is not much altered by changing the size of antigen dose, while γG antibodies appear considerably sooner when larger

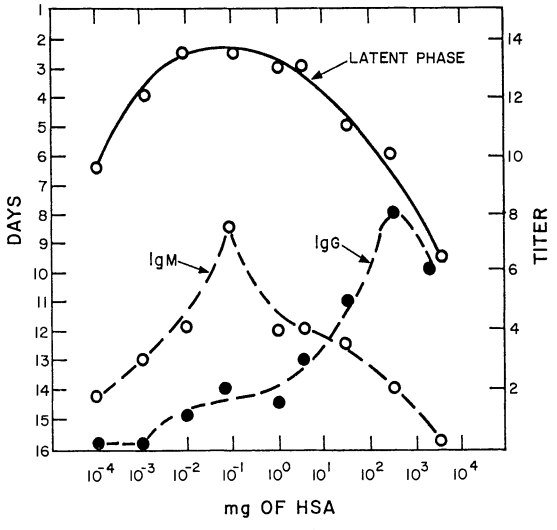

Fig. 3. Peak serum titres of IgM and IgG antibodies and duration of latent phase in chickens injected with various doses of HSA

doses of antigen are given (SVEHAG and MANDEL, 1964b). An increase in the dose of antigen resulted in shortening of the half life of both γM (from 16 to 6 hours) and γG (from 72 to 6 hours) antibodies (UHR and FINKELSTEIN, 1963, 1967; SVEHAG and MANDEL, 1964a).

IVÁNYI et al. (1966) and VALENTOVA et al. (1967) using a dose range of HSA in chickens from 0.1 μg to 5 g per kg weight obtained the whole theoretical curve of the relationship between the antigen concentration and the immune reaction, starting with the threshold dose of antigen which stimulated antibody synthesis and ending with the inhibitory supraoptimal dose. Maximal γM and γG titres and the duration of the latent phase after various doses of antigen are compared in Fig. 3. In the region of smallest doses only γM antibodies were produced and on raising the dose, the latent period was shortened. With medium doses of HSA γG antibodies appeared, the latent period was prolonged and the γM titre decreased. After the highest used doses no γM antibodies were produced and the γG response was also progressively delayed and diminished. These results indicated that the γM antibody response has a lower threshold for inhibition as well as for induction, so that it can be selectively inhibited without alteration of the γG antibody response.

The significance of serological studies in the determination of the kinetics of synthesis of different molecular types of antibodies is limited by several factors: a) degradation of antibodies by different half-lives; b) formation of antigen-antibody complexes at various extent; c) differences in the sensitivity of the serological methods. Particularly in situations where antigen circulates for a longer period, the early rise in antibody synthesis can be altered by the choice of serological methods. Nevertheless maximum antibody titres, which are not attained for a few or several days, are a relatively objective criterion of total stimulation.

The increase in the proportion of γG antibodies after large doses of antigen does not apply with all antigens. When the dose of KLH in rabbits was raised from 0.1 to 2 mg the maximum titre was raised and was attained sooner, but a further increase in the dose from 2 to 1,000 mg did not alter the titre (DIXON et al., 1966). However, the relative proportions of γM and γG antibodies at any given time after immunization remained constant over this wide range of dose, with the γM antibodies always appearing before the γG. It seems that these findings, especially the prolonged synthesis of γM molecules may be in part due to rapid elimination and degradation of KLH (within 6 hours of intravenous administration), so that differentiation of the cells and the maturation of new competent cells does not take place under the direct influence of extracellular antigen.

The avidity of antibodies, which increases with time after immunization (JERNE, 1951), was studied by STEINER and EISEN (1966) in relation to the antigen dose. An increase in the antigen dose delayed the change in the affinity of antibodies. Presuming, that antibody synthesis takes place only when the antigen concentration is within fixed limits, it was suggested, that for each cell these limits are related to the affinity of the antibody produced. With a high antigen level, only cells producing low-affinity antibodies are stimulated and vice versa. High-affinity cells are not active when their upper threshold for stimulation by antigen is exceeded, but escape from this "inhibition" as the antigen concentration diminishes.

b) Cellular Studies

The use of methods which detect antibody producing cells directly (COONS et al., 1955; JERNE and NORDIN, 1963; INGRAHAM and BUSSARD, 1964; BAKER et al., 1966) confirms the results obtained in serological studies. Using the plaque technique, JERNE et al. (1963) and JERNE (1965) found that after increasing the immunizing dose of sheep RBC given to rabbits the latent period, which occurs before plaque-forming cells appear in the spleen, was shortened from 48 to 24 hours. At the same time the rate of accumulation of plaque-forming cells rose and more cells were obtained sooner. The system had a saturation level, as doses of more than 4×10^7 sheep RBC did not alter the accumulation curve and the total count of plaque-forming cells. Similar results were obtained by WIGZELL et al. (1966) who found, that as the dose of sheep RBC

was raised, and the accumulation rate (T_2) was shortened from 11.7 to 5.4 hours. The latent period remained about 24 hours over the dose range studied. The curves of direct plaque-forming cells which can be regarded as cells producing IgM haemolysins fell rapidly after reaching maximum (Wigzell et al., 1966; Hege and Cole, 1966). A modified method which permits the separate demonstration of IgG-producing cells (Dresser and Wortis, 1965; Šterzl and Říha, 1965) was used for antigen dose — response studies by Wortis et al. (1966). They found at 3 weeks after immunization that the number of plaques developed by anti-IgG serum continued to be directly related to antigen dose, while the number of direct plaques for all doses had by this time reached a plateau. They have suggested, that the antigen requirements for log-continued production of the two classes of cells are different. Furthermore they found, that the region where a small increase in antigen dose effects a more than equivalent increase in plaque-forming cells is attained with lower doses for direct (IgM) than for developed (IgG) plaques. Baker and Landy (1967) have studied the cellular reaction after immunization with different doses of bacterial antigens, using for detection of antigen-reactive cells their adherence to bentonite particles coated with the relevant antigen. The pneumococcal polysaccharide and E. Coli lipopolysaccharide which they used are only metabolized with difficulty by the reticulo-endothelial system in the animal and they might therefore be presumed to influence the antigen — sensitive cells directly. When the immunization dose of this type of antigen was increased, the latent phase, which in general is much shorter than in other antigen systems, was reduced. After optimal antigen dosage for stimulation it was less than 60 minutes (found after extrapolation) and the T_2 during the first 24 hours of the reaction was 5.4 hours.

The indirect immunofluorescence method was used by Černý and Iványi (1967) for the study of cellular response in the spleen of chickens immunized with different doses of HSA. Although it can be assumed, that this method is less sensitive since it is impossibile to examine all the cells in the organ, the results were very similar to those obtained by the haemolytic plaque technique. It was possible to define the limits of the size of immunizing doses from the threshold (inducing) dose to the supraoptimal (inhibitory) dose (Fig. 4). On raising the immunization dose from 1 μg to 5 mg HSA, the duration of the latent phase was reduced, the T_2 of antibody producing cells was *shortened* and their number increased. When the dose of HSA was raised to 500 mg, the latent phase was *prolonged* and the first antibody producing cells were not detected until after 72 hours. This delay in the onset of the appearance of antibody producing cells which occurs on raising the antigen dosage in certain region was interpreted at serological level (section 3 a) as being a manifestation of the predominance of an IgG reaction and/or selective inhibition of IgM synthesis (Iványi et al., 1966). In this connection it is of interest that during the response to high doses of HSA the fluorescence positive cells are predominantly mature plasmacytes, while less mature lymphoid cells predominate at the peak of the reaction to small doses of antigen (Černý et al., 1965).

With low doses of antigen the rate of increase (T_2) of the exponential phase shortens as the dose increases. When a given limit is reached however, larger doses stimulate the immune response only by shortening the latent period, while the T_2 remains unaltered (ALBRIGHT and MAKINODAN, 1965; UHR and FINKELSTEIN, 1967). It therefore looks as if antigen primarily regulates the rate of division of a small initial number of antigen-sensitive cells, and secondarily and mainly at higher doses an effect which consists of the inactivation of an

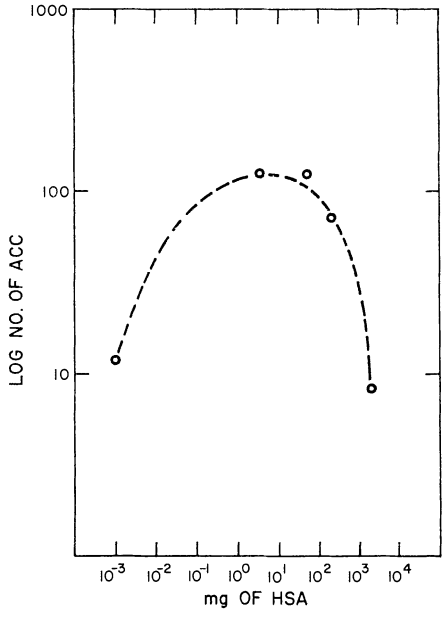

Fig. 4

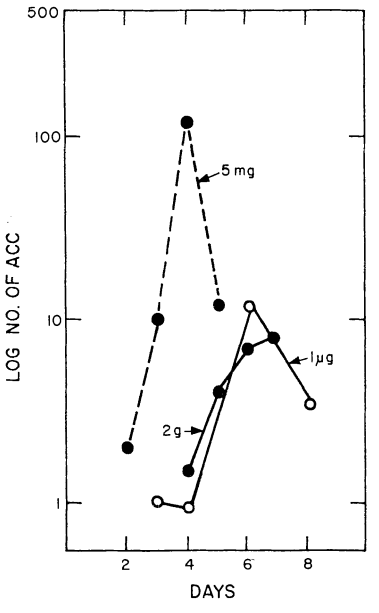

Fig. 5

Fig. 4. Peak numbers of antibody containing cells (*ACC*) detected by immunofluorescence in spleen imprints of chickens injected with various doses of HSA

Fig. 5. The kinetics of antibody containing cells (*ACC*) in the spleen of chickens injected with a low (1 µg), medium (5 mg) or high (2 g) dose of HSA

increasing number of new cells. Experimental evidence in support of this hypothesis can be adduced from the experiments of ALBRIGHT and EVANS (1965). They showed that in a cell transfer system, increasingly large innocula of primed cells plus antigen resulted in a shortening of the latent period and also in higher titres of serum antibody but no change in the rate of increase of antibody titre (T_2) was seen. When the dose of antigen exceeds the saturation level, the reverse process is observed: the latent period is prolonged, antibody-producing cells accumulate at a slower rate and finally the antibody response is inhibited (MAKINODAN et al., 1965). ČERNÝ and IVÁNYI (1967) showed, that when the dose of HSA in chickens was raised to 2,500 mg, the rate of accumulation and the number of antibody producing cells ceased, producing a curve similar to that obtained after immunization with a threshold dose (1 µg HSA) of antigen (Fig. 5).

To sum up, we find that the shortest experimentally determined T_2 for antibody producing cells (about 5 hours) confirms the view of INGRAHAM (1964) that the initial exponential increase in the antibody titre cannot be explained on the basis of the division of antibody producing cells, as the generation time of lymphoid cells is reported to be about eight hours (CAPALBO and MAKINODAN, 1964; SADO and MAKINODAN, 1964). Moreover some authors have demonstrated that a large number of antibody producing cells had not synthesized DNA under used experimental conditions (COHEN and COHEN, 1964; ŠTERZL et al., 1965; TANNENBERG, 1967). It is far more probable that the rapid increase in the antibody titre is the outcome of differences in the point at which individual cells start to produce antibody and of cuccessive accumulation of cells which are synthesizing antibody after periods of cellular differentiation of varying lengths of time (INGRAHAM, 1964; ŠTERZL et al., 1965). We could therefore assume that the number of antibody producing cells, the rate of their differentiation and the relationship of this rate to antigen concentration are simply related to the number of "sensitive" cells stimulated initially. In the simplest possible model, such a stimulation of sensitive precursor cells would be an all-or-none phenomenon. In a heterogenous population most of the cells would be stimulated by the optimal dose of antigen, while fewer would be stimulated by smaller or larger doses. This might explain the similarity of the course of differentiation of antibody producing cells after a small and after a large dose of antigen (Fig. 5). The formation of a large number of antibody producing cells by direct differentiation from progenitor cells inevitably presupposes progressive maturation and activation of newly produced antigen-sensitive cells. According to the calculations of MAKINODAN and ALBRIGHT (1962), JERNE (1965) and ŠTERZL et al. (1965), very few cells which react with antigen are actually available at the time of immunization (about 500 in a mouse). However, as antigen also stimulates the division of cells (MAKINODAN and ALBRIGHT, 1967), factors other then cell division may be involved in the cytokinetics of antibody formation.

c) Studies with Isolated Cells

A well defined model of antigen dose mediated regulation of the differentiation of lymphoid cells is to be found in experiments using cultivation of cell suspensions either in the milieu of a syngeneic irradiated organism, in an intraperitoneally implanted diffusion chamber or in culture *in vitro*. These models simplify the situation of whole organism in some respects and permit variation of the antigen: cell ratio.

The model of *in vivo* transfer has been elaborated from the aspect of the antigen dose mainly in the Oak Ridge laboratory, where cells from normal animals or from animals preimmunized with heterologous red blood cells were transferred to irradiated syngeneic hosts, and the haemagglutinin titre then measured (reviewed by MAKINODAN and ALBRIGHT, 1967). Within a given size range of the cell inoculum, the log of the antibody titre in the host is a linear function of the log number of transferred cells. Following an increase in the

ratio of antigen to primed spleen cells, the latent phase of the immune response was shortened, the peak titre was increased and attained at a higher rate. At the ratio of antigen to primed cells of 100 and over, the reverse process occured: the onset of agglutinin formation was delayed, the rate of titre increase was slow and the peak titre values were decreased. The curve showing the relationship of the maximum titres to the antigen dose was parabolic, being similar to that obtained by measuring the cellular reaction *in vivo* (section 3 b). A possible theoretical interpretation of these results is, that within the extremes of an antigen dose range, a change in the antigen concentration is probably reflected by a change in the number of active progenitor cells as well as a change in the number of antibody producing progeny formed from a single progenitor cell. The possibility of a change in the generation time of functional progeny must also be considered. However ALBRIGHT and EVANS (1965) and ALBRIGHT and MAKINODAN (1965) regard the amount of antibody synthesized by any one antibody-producing cell as the main variable factor.

The results mentioned above were all obtained after stimulation of presensitized (PC_2) cells. It is interesting that the subsequent immune response was inhibited by high antigen concentration only in these PC_2 cells and not in non-committed (PC_1) cells (ALBRIGHT and EVANS, 1965). However BUSSARD and ANDERSON (1966) using diffusion chambers for cultivation, have reported at high antigen (sheep RBC) to normal mouse spleen cell ratio inhibition of agglutinin formation, but no inhibition of haemolytic plaque-forming cells or haemolysin titre in the chamber.

Some complex ecological factors could also participate in cultivation *in vivo* as indicated by the finding of "premium effect" by CELADA (1967). He found that the antibody titre in the recipients' serum was not linearly proportional to the number of cells transferred and rose to values higher than would be expected from their number. For example a 10-fold increase in the size of cell inoculum was accompanied by a 60-fold increase in the titre. The "premium effect" was observed over a wide range of antigen dose (about 10^4 fold); on further raising the challenge dose, however the immune response was depressed, particularly with the maximum cell dose (2×10^7) used.

Experiments in which primed spleen cells were cultivated with antigen in a diffusion chamber gave similar results to *in vivo* transfer experiments (MAKINODAN et al., 1965). A parabolic curve of the correlation of the antibody titre to the antigen dose was demonstrated and in addition, the rate of change in titre after small suboptimal dose of rat erythrocytes was similar to the curve after a supraoptimal dose, as was found in experiments in whole animals (Fig. 5). The recovery of cells from the chamber permitted autoradiographic study of the synthesis of RNA, DNA and proteins (MAKINODAN et al., 1965). Their metabolism was influenced by the dose of antigen in a similar manner to the final antibody titre: it was stimulated after an optimal dose and depressed after a supraoptimal dose. This was manifested as a change in the number of cells labelled with radioactive precursors. The number of grains — a measure of the activity in a single cell — was independent of the antigen dose, which

provided evidence in support of an "all-or-none" phenomenon in the process of induction at the level of a single cell. Because of some technical problems involved, it is important that undue weight should not be attached to any one part of the evidence.

In vitro experiments are concerned chiefly with preimmunized cells. Dutton and Eady (1964) found that the addition of antigen to preimmunized cells in vitro led to an increase in the incorporation of ^{3}H-thymidine into DNA and that the degree of incorporation was proportional to the antigen concentration. Stimulation with cross-reacting antigen evoked a similar relationship (Dutton and Page, 1964). Brownstone et al. (1966) investigated the question of the significance of homology of the carrier of the hapten in induction of the secondary response. They found that the apparent lack of cross-stimulation by the hapten when conjugated to heterologous protein could be overcome by the use of sufficiently large doses of conjugate. Cells primed with NIP-ovalbumin were relatively insensitive to stimulation with NIP-BSA, unless 10^4 more NIP-BSA was used.

The experiments of Dutton and his colleagues did not demonstrate the inhibition of DNA synthesis in primed cells with high concentrations of antigen. This conflicts with the findings of Mäkelä and Mitchison (1965) and Caron (1967), who also measured incorporation of ^{3}H-thymidine into DNA. They found an initial increase, followed by a depression of activation of DNA synthesis as the antigen concentration was raised. Similarly Benezra et al. (1967) found that a supraoptimal antigen concentration inhibited blast transformation. If the experiments mentioned above are measuring the state of delayed-hypersensitivity of the population of cells under study, as has been suggested by Mills (1966), then they do not necessarily have any direct bearing on the processes involved in humoral immunity.

Diener and Armstrong (1967) recently published a detailed study on induction of the primary response *in vitro* with reference to the concentration of antigen. By cultivating cells on a membrane filter they detected an inhibition of the cellular immune reaction to high concentrations of S. adelaide flagellar antigen. They described a plateau (evidently the optimal dose of antigen) and the descending part of the curve of antigen dose — response relationship, but did not find a phase in which the response rose parallel with the antigen dose.

4. Immunological Memory

According to the calculations of Makinodan and Albright (1967) a preimmunized organism contains about 100 times more antigen-reactive cells than a non-sensitized organism. Comparison of the threshold antigen concentrations needed for induction of the primary and secondary antibody responses ought to provide information on the significance of the proportion of the number of antigen reactive cells to the concentration of antigen. It is impossible to draw a general conclusion from their results however, as the study of another model systems has produced completely different results. Nossal

et al. (1965a) found in rats immunized with Salmonella adelaide flagellar antigens that the dose of antigen needed to induce a secondary response was of the same size, or larger than the dose needed for primary immunization. Controversly, in recent experiments by ŠTERZL and JILEK (1967) with erythrocytic antigen in mice, the threshold antigen dose for initiation of the secondary response was considerably lower than the dose needed for primary immunization.

The significance of antigen dose for the molecular type of antibodies in the secondary response was first demonstrated by UHR and FINKELSTEIN (1963) and SVEHAG and MANDEL (1964b). These authors failed to induce an increased secondary response following a primary IgM response at the time of challenge and interpreted their results as indicating that the cells responsible for γM antibody synthesis exhibited no immunological memory. However extensive studies of NOSSAL et al. (1965a) demonstrated that pronounced memory of the IgM type could be induced by a small dose of antigen. Secondary IgM response was found also by other authors (NEZLIN, 1965; PORTER, 1966; VALENTOVA et al., 1966). VALENTOVA et al. (1967) have analysed the conditions under which IgM memory could be demonstrated and found, that they differed considerably from those leading to secondary response of the IgG type. Their experiments showed that the two critical conditions which determine the type of antibodies produced in the secondary response were the size of the antigen dose used for primary immunization and the time interval between primary and secondary immunization (Fig. 6). Immunological memory of the IgM type, using small doses of antigen, reached the maximum a few weeks after immunization and decreased then rapidly. However, the secondary IgG response after stimulation with larger doses of antigen (about 10^4 times), could be induced only after a longer time interval and persisted for several months. The rapid onset and cessation of IgM memory and the longer latent period of IgG memory are reminiscent of the kinetics of production of these two types of antibodies in the primary response. The differences in the kinetics and antigen dose requirements of IgM and IgG memory indicate, that the antigen dose plays a regulative role in the cytokinetics of memory cells and that at least 2 populations of memory cells exist, each with a potential for producing different molecular types of antibodies.

With a large dose of antigen, which induced a maximum IgG response a challenge injection given soon after primary stimulation led to inhibition of γM antibody formation (IVÁNYI et al., 1966; VALENTOVA et al., 1967). A comparable situation was observed in the immune response to SRBC: with higher primary antigenic stimuli leading to higher primary responses, lower secondary responses were found (ŠTERZL, 1966; JILEK and ŠTEZL, 1967).

Data concerning the relationship between antigen dose and the rate of antibody production in a primary and secondary response provides evidence for the existence of a line of "memory" cells differing from the cells responsible for actual antibody production. For example no direct relationship between the size of the primary antibody response and the size of a subsequent memory

response has been found: in chickens after a dose of 0.1 μg HSA, far less antibodies are formed than after a dose of 10—100 μg, but the size of IgM secondary response induced after such doses is the same (VALENTOVA et al., 1967). The reverse disparity was found by NOSSAL et al. (1965a) in whose system, after a given point had been reached, further elevation of the immunizing dose of antigen did not enhance primary antibody formation,

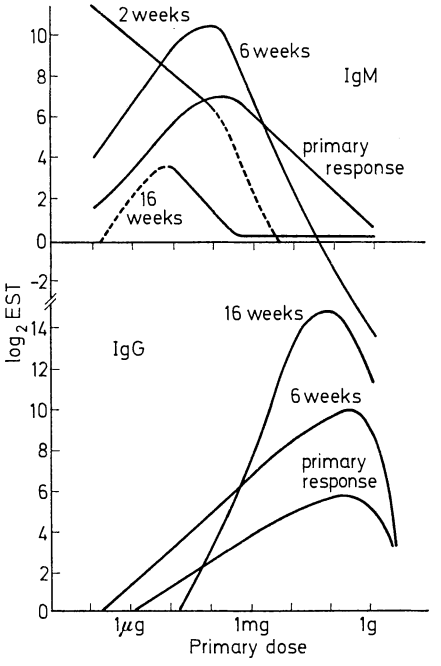

Fig. 6. Extrapolation of IgM and IgG peak excess secondary titres (*EST*) in chickens preimmunized with various doses of HSA and challenged after 2, 6, or 16 weeks with 20 mg HSA. (From VALENTOVÁ et al., 1967)

although a subsequent 2° response continued to increase with increasing dose of antigen.

The formation of a line of memory cells can be visualized in different ways. One hypothesis is that only one contact with antigen is necessary for the priming of competent cells, while two are required for the stimulation of antibody synthesis (LEDUC et al., 1955; SERCARZ and COONS, 1962). A small dose of antigen is sufficient for "one hit stimulation", while a larger dose also provides sufficient antigen for a second contact, leading to differentiation of the cells into the productive phase. This concept supplies a satisfactory explanation for the experimental phenomenon, that priming can be induced without a demonstrable primary immune response (NOSSAL, 1965a). This hypothesis is also compatible with evidence from the enhanced response to antigen administered as early as the 2nd or 3rd day after immunization (SERCARZ and BYERS, 1967; POSPIŠIL and HAJEK, 1967). These experiments may explain the older finding that successive daily injections of antigen can

elicit a higher antibody response than the same amount given in a single dose (CARLINFANTI, 1951). On the other hand the rise in potential of the immunological memory (FECSIK et al., 1964; BLAZKOVEC and WOLFE, 1965) with time and the delay in its development, with increasing doses of antigen (VALENTOVA et al., 1967) is not explained. The results suggest that the decision as to whether the cell is stimulated without production (memory), or whether it proceeds directly into the production phase, does not depend solely on the number of hits by antigen. These 2 hypothetical types of differentiation of individual cells might take place during the first contact of a heterogeneous cell population: the form that the resulting differentiation takes being dependent on the effective concentration of antigen in the cell concerned. Reciprocal regulative processes between cells which have already been stimulated and stem cells still in process of differentiation might affect the immune response, as could also the antibody feedback effect, which is discussed elsewhere (section 6b).

5. Immunological Tolerance

a) Induction, Maintenance and Loss

The term immunological tolerance, paralysis or unresponsiveness was used in a broad sense to define several forms of specific immunological non-reactivity induced by the exposure of adult or immature animals to a potential antigen. The phenomena covered by this definition represent a wide range of experimental observations, which have been discussed in several reviews (HAŠEK et al., 1961; SMITH, 1961; DE WECK and FREY, 1966; DRESSER and MITCHISON, 1968; and HRABA, 1968). The experimental results clearly demonstrate a relationship between the duration of tolerance and the size of the initial dose or the level of persisting antigen in the animal. It is difficult to decide whether the dose of antigen is more relevant to the establishment of a tolerant state or to its duration. The criteria for the quantitative measurement of both induction and maintenance overlap. Since the process of the induction of tolerance usually takes several weeks and a certain time after induction is usually chosen for its testing, determination of the direct effect of antigen cannot be restricted only to the reactive cell population, but it must be presumed that it also includes new cells in process of differentiation from stem cells (MITCHISON, 1962a). The rate of induction of, and escape from tolerance provide evidence, that prolonged persistence of antigen which interferes in some way with the participation of newly differentiated immunologically competent cells in the induction of immunity is required for the induction of long-term tolerance. Although it has been discussed recently, that the amount of antigen is only of importance where the paralysing antigen induces a concomittant state of immunity (DRESSER and MITCHISON, 1968).

A comparison of antigen dose requirements for induction of tolerance to various antigens is difficult, as few experiments are done in the same animal species under comparable conditions. In most cases, however, one injection

of antigen is not sufficient to induce inhibition and it is therefore administered at closely spaced intervals over a period of several weeks. There are considerable differences between species when protein antigens are used. Tolerance induced in newborn rabbits by a single injection of heterologous albumin persists for several months or even for more than a year (Smtih, 1960), while in chickens tolerance even to a relatively weak antigen (duck albumin) disappeares within six weeks after the supply of antigen is discontinued (Iványi and Valentova, 1966).

Persistence of antigen seems to be a necessary condition for maintenance of an unresponsive state. Tolerance to RBC can be broken by removing the antigen from the blood stream by means of passively administered antiserum (Mitchison, 1962b; Hašek and Puza, 1962). Tolerance to protein antigens cannot be abolished by antiserum (Dixon and Maurer, 1955), but Brooke (1964) could partially abolish tolerance to pneumococcal polysaccharide S III by means of the specific depolymerase enzyme a few days after induction. This suggests that the intracellular antigen may be sequestered or protected in some as yet unexplained manner. Dietrich and Weigle (1963) found in mice that the induction and duration of tolerance to various heterologous proteins was not in agreement with their half-life of degradation. For these reasons, calculations of the amount of antigen for maintenance of tolerance based on the half-life of circulating antigen (Smith, 1960) probably do not contribute to an understanding of the phenomenon. Humphrey (1964b) demonstrated that following neonatally induced tolerance to HSA in rabbits, the capacity to respond to the different parts of the antigenic mosaic returned piecemeal. It was assumed that if paralysed cells, which are temporarily blocked by antigen, persist, then the separate antigen determinants required for each potential specificity must also persist for varying lengths of time. Alternatively, if responsiveness is achieved by replacement with new cells, their capacity for responding to different parts of the antigen ought, in the early stages, to be limited.

It can be assumed that when the antigen concentration in the tolerant animal falls below a given critical level, antibody formation is no longer inhibited and the antigen still remaining in the animal can act as a stimulus. Evidence for either spontaneous or increased immunity ("overshoot") was submitted in several studies in mice (Terres and Hughes, 1959; Thorbecke et al., 1961; Sercarz and Coons, 1963). However studies in rabbits failed to support these results (Smith, 1961) and in ckickens only in some individuals when recovered from partial tolerance, and increased response after challenge was found (Iványi and Valentova, 1966). Immunity found after escape from tolerance may probably be the outcome of concommittent immunization by the paralyzing injections of antigen.

Once induced, tolerance can be maintained by a small dose of antigen, considerably lower than the dose required for the actual induction of tolerance. Smith (1960) showed that the duration of tolerance to bovine albumin in rabbits was significantly prolonged by the "immunization" dose of antigen

used to test tolerance. "Maintenance" antigen doses could either help to replenish the antigen concentration at any given site to the value needed, or could help induce inhibition in newly differentiated cells. It can be assumed that susceptibility to antigenic stimulation changes during escape from tolerance. It was found (IVÁNYI and VALENTOVA, 1966; DOWDEN and SERCARZ, 1967) that the tolerance-maintaining effect of middle range antigen doses was obtained only if they were injected sufficiently early, i.e. when tolerance was complete, whereas in disappearing tolerance it may act as an immunizing stimulus. It might be concluded that the injection of antigen during a state of partial tolerance could have two effects: 1) acceleration of recovery of the immune response if the degree of tolerance was weak before antigen was administered, 2) prolongation or even reinduction of tolerance (HAŠEK and PUZA, 1962; HUMPHREY, 1964a) if the degree of inhibition was sufficiently strong before antigen was administered.

b) The Mechanism of Induction

The mechanism of the interaction of antigen at a high concentration with a population of antigen-reactive cells is discussed in this section. The mechanism of inhibition of responsiveness is a complex phenomenon, especially when it is borne in mind that within certain dose limits the animal's immune reaction increases as the dose of antigen is raised .In some situations it is evidently impossible to inhibit directly the immune reaction of antigen-reactive cells, however high the dose of antigen used may be. In this respect, experimental results were furnished by SIMONSEN (1960, 1962), who transferred parental lymphoid cells to newborn F_2 offspring, where they induced splenomegaly, followed by tolerance of the donor cells. Simonsen termed this phenomenon "exhaustive sensitization" and concluded that after a sensitization phase the immunologically competent cells were inhibited. Similarly, it was found that inhibition could not be obtained directly by primary stimulation with strong erythrocyte antigens; this led to the view that tolerance can be induced in an adult animal by a saturation dose of antigen only by the exhaustation of immunologically competent cells via a productive phase (ŠTERZL et al., 1966). When comparing these views with experiments demonstrating direct inhibition as the outcome of the primary interaction of antigen with competent cells (described below), critical differences between the models should first of all be pointed out. In the case of heterologous RBC, in a dose-response study with isolated lymphoid cells it was calculated (MAKINODAN et al., 1965) that the minimum cell dose which led to only partial inhibition of the reaction in the isolated system was equivalent to the in vivo administration of 10^{11}—10^{12} RBC per mouse. The doses needed to produce deeper or complete inhibition of the response would thus exceed the limiting maximal quantity or red cells, which can be injected into a mouse. Another factor emerges from the experiments of COHEN and SAPP (1967), who found that the degree of cellular eosinophilic reaction is decided not by the *weight* concentration, but by the *molar* concentration of the antigen injected. Although we do not know what molar concentra-

tion of erythrocytic antigens can be reached in the organism, it can be assumed that concentrations of several orders higher can be obtained by the use of protein antigens.

In experiments with HSA in chickens it was found that daily injections of 1 g HSA per kg body weight gave a saturation concentration of antigen in the blood stream (IVÁNYI and ČERNÝ, 1965) which inhibited the appearance of antibody-containing cells demonstrable by the immunofluorescence technique (ČERNÝ and IVÁNYI, 1967). Further studies (ČERNÝ and IVÁNYI, 1966) indicated, that: a) an initial "tolerogenic" dose of antigen if maintained by repeated injections of antigen does not induce antibody synthesis, but leads directly to its inhibition; b) in some of the competent cells the immune differentiation induced by an immunizing dose of antigen begins so rapidly, that after 24 hours it can no longer be inhibited by a tolerogenic dose of antigen; c) the indirect immunofluorescence technique does demonstrate antibody-containing cells in the excess of extracellular antigen. Similar results were obtained by DIENER and ARMSTRONG (1967), who succeeded to supress the primary immune response in vitro, using a supraoptimal concentration of flagellin polymer antigen. BAKER and LANDY (1967) also obtained complete inhibition of antigen-reactive cells after the injection of large doses of bacterial polysaccharide in vivo. In their system, however, the addition of antigen to a spleen cell suspension led to inhibition of the adherence of antigen-coated bentonite particles, so that they could not exclude the possibility that inhibition might have been caused by masking of the reaction by antigen.

Further evidence on the mechanism of tolerance by protein overloading comes from experiments in which the spleen fragments of chickens injected with various doses of HSA were cultivated in vitro with or without actinomycin D in the culture medium (IVÁNYI et al., 1968). On using optimal antigenic stimulation an early (2nd day) actinomycin D-sensitive antibody synthesis was observed, followed by actinomycin D-resistant antibody synthesis in cultures from spleens removed later (5th day) after immunization. As the antigen dose was raised, the period of actinomycin D-sensitive antibody synthesis was prolonged, suggesting that the synthesis of antibody-specific RNA was delayed. On further raising the antigen dose, there was a delay in the onset of detectable antibody synthesis in vitro and recovery took place only in the absence of actinomycin D. Both experiments indicated that processes which precede antibody synthesis — such as RNA synthesis — are delayed by the increasing dose of antigen. Another evidence about the controlling role of antigen dose for specific RNA synthesis was presented by PAGOULATOS (1965). We consider the phenomenon of "delay" of immune differentiation after a large supraoptimal dose of antigen described earlier in serological and cellular terms (section 2, 3a and 3b) as a basic condition for direct inhibition of the antigen-reactive cell population. The necessity of maintenance of the limiting concentration of antigen in the animal for a certain time, to acchieve long-term tolerance is probably determined by the rate of repopulation of new antigen-reactive cells from stem cells.

c) Nonspecific Processes

The antigen concentration certainly affects "non-specific" cellular changes of lymphoid organs and "non-antibody" globulin synthesis of animals. It seems evident that not all the cells differentiated as a result of injection of antigen produce specific antibody and that serum immunoglobulin synthesis can increase. Induction of tolerance in the high antigen dose range is of interest here, because selective inhibition of the specific component of the reaction, together

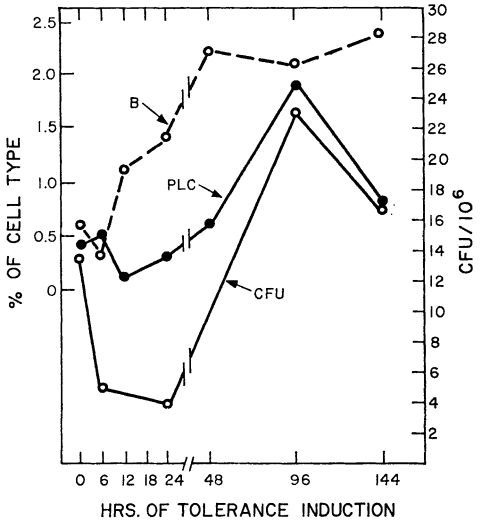

Fig. 7. The kinetics of blast cells (*B*), plasma cells (*PLC*) and colonyforming units (*CFU*) in the spleen of adult mice during the induction of tolerance with daily massive doses (1 g/kbw) of HSA

with stimulation of nonspecific processes occurs. ČERNÝ and VIKLICKY (1967, 1968) induced tolerance in mice by daily injections of immunochemically pure HSA monomer and found within the first 6—12 hours in the spleen a depletion of the red pulp, cell disintegration in the centre of the white pulp follicles and a decrease of gamma-globulin containing and colony-forming cells (Fig. 7). On subsequent days the hypertrophy of the white pulp developed, but as distinct from immunized mice, the appearance of secondary germinal centers and cellular infiltration of the red pulp was absent. The number of blast cells, plasma cells, gamma globulin containing and colony-forming cells increased (Fig. 7). Moreover a 3—4 fold increase in the number of haemolytic plaque-forming cells against sheep RBC was found. All these changes occured under conditions, when the specific immune response to HSA was absent. However, these nonspecific reactions can evidently occur during induction of immunity, but are more pronounced when tolerance is induced. HANNA et al. (1966) found that the germinal centre reaction, early after immunization, increased in intensity and duration with rising the dose of heterologous RBC, in a range nowhere near specific inhibition. Cellular replacement may be of significance, because an inhibition of cellular reaction by a high concentration of antigen

can be obtained when isolated cells are used (section 3 c) and probably in the draining lymph node after local injection of antigen (Zaleski et al., 1966).

Another changes during protein-overloading of guinea pigs was described by Liacopoulos and Neveu (1964), Liacopoulos and Good (1964) and Liacopoulos et al. (1967). They described a temporary depression of the immune reaction to unrelated protein or tissue antigens and an increase in the concentration of serum gamma$_1$ and gamma$_2$ globulins. Gamma$_2$ globulin production was proportional to the total amount of antigen administered.

The data about the "nonspecific" response induced by challenge injection of antigen in already immunologically tolerant animals vary. An absence of histological changes in lymphoid organs was reported by Cohen and Thorbecke (1964) and Gitlin et al. (1958). Dutton (1964) found an absence of antigen induced DNA synthesis of spleen cells in vitro. However, Černý and Viklicky (1968) have reported in mice an increase of blast cells in the spleen if a large dose of the tolerated HSA was used for stimulation. Hanan and Oyama (1954) have found after challenge increased serum gamma globulin levels in the absence of antibody formation in rabbits tolerant to HSA. Schechter (1967) found, that competitive inhibition of reaction against a hapten by another hapten-carrier complex occured in animals tolerant in respect the competitor hapten. Harris (1967) showed that macrophages from tolerant donors were fully reactive as far as their stimulant effect on primed lymphoid cells was concerned, after previous treatment with antigen. The list of evidence given, allows us to conclude that we should admit the existence of some forms of recognition and interaction between antigen and cells, which are not associated with formation of specific antibodies in immunologically tolerant animals.

6. Tolerance Phenomena induced by low Doses of Antigen

a) Non-immunogenic Antigens

Attempts to induce unresponsiveness by minute amounts of antigen were stimulated by the work of Dresser (1962) who demonstrated that BGG deprived of aggregated molecules by ultracentrifugation induced tolerance in CBA mice. He suggested that the antigen molecule requires some adjuvant properties for the induction of immunity and that in their absence antigen induces tolerance. Using an appropriate schedule of injections (exponentially decreasing amounts of BGG) tolerance could be induced also in immunized mice (Dresser, 1965). Dresser and Gowland (1964) also demonstrated this phenomenon in rabbits and calculated that 10^8—10^9 antigen molecules were needed for its maintenance, i.e. 100 times less than in CBA mice (10^{11}—10^{13} molecules). With a non-immunogenic antigen such as BGG, tolerance can be induced with a single injection, while the induction of tolerance to an antigen such as BSA, for which "adjuvanticity" is an integral property, always requires a series of injections. For this latter antigen Mitchison (1964) demonstrated in mice a new phenomenon — the existence of two zones of antigen

dosage where specific inhibition of the immune response occured; intermediate doses of BSA led to immunity. He suggested that the two thresholds of immunological unresponsiveness were determined by the ability of virgin immunologically competent cells to undergo induction in one of two directions — towards immunity or towards tolerance. The potentially reactive cell population might be heterogeneous, with most cells becoming tolerant after high antigen concentrations, but some being immunized. In this respect, concomitant immunization would be an obligatory event in partial tolerance. In cells initially comitted in the direction of immunity, the threshold antigen concentration required to induce tolerance is increased, that is to say, if a high antigen concentration persists, it can inhibit previously immunized cells. This course of events is in agreement with the hypothesis (DRESSER, 1962, 1965) which postulates that immunologically competent cells have two types of specific sites for induction of either immunity or paralysis, and that intracellular antibody controls the amount of antigen required for saturation of the paralysis site. According to THORBECKE and BENACERRAF (1967) there are two possible explanations for low dose paralysis: 1. Conversion of stem cells into tolerant cells, which after prolonged contact with antigen replace the population of mature antigen sensitive cells without stimulation of the latter; 2. it may be the outcome of exposure of immunologically competent cells to antigen which, because of lack of adjuvanticity has not been previously processed by macrophages (FREI et al., 1965).

b) Immunogenic Antigens

The extent to which the phenomena described above can be compared with inhibition states after prolonged administration of small doses of strong immunogens or particulate antigens is not clear. Inhibition of the immune response after repeated immunization with heterologous RBC was described in rats (NEIDERS et al., 1962). Similar inhibition was observed in rabbits (GRAS and DALMAU, 1966), however if in the course of the injections of small doses of RBC a single 10 times larger dose was administered, it was followed by an abrupt hyperimmune reaction. ROWLEY and FITCH (1964) suggested that by small doses of heterologous RBC only part of the immunologically competent cell population was stimulated and that the low level of antibody synthesis is under the threshold of detection. They postulated that the formation of small quantity of antibodies could inhibit the reaction of the remaining uncomitted cell population by a feedback inhibitory mechanism and that the inhibitory effect of antibodies requires their direct interaction with immunologically competent cells. Further evidence of similar mechanisms was obtained in chickens injected daily for 2 weeks with microgramme amounts of HSA (IVÁNYI, unpublished results). A diminished immune response was found in chickens, which had low titres of antibodies before challenge by HSA. Another view is, that the main effect of antibody in inhibiting the immune response is neutralization of the immunogen (MÖLLER and WIGZELL, 1965). It was suggested however, that inhibition is caused by neutralization of antigen, which has

already been incorporated into immunologically competent cells and that interaction does not necessarily take place with the whole antigen molecule, but at the level of single antigenic determinants (Dixon et al., 1967). Further evidence that passively administered antibody blocks immunogenicity of "processed" antigen is supplied by the finding that the induction of an anamnestic response is not inhibited (Rowley and Fitch, 1964).

c) Deviation

The injection of a given dose of antigen leads in some experimental situations to selective inhibition of only some types of immune reactions of the animal. The injection of a protein antigen in solution a few weeks before, or simultaneously with stimulation in complete Freund's adjuvant has been found to inhibit delayed hypersensitivity in guinea pigs (Asherson and Stone, 1965; Dvorak et al., 1965; Jones and Leszkowitz, 1965; Borel et al., 1967). Synthesis of humoral antibodies was only partly inhibited in these experiments; 7 S gamma$_2$ antibodies were inhibited to a greater degree than) 7 S gamma$_1$ antibodies. This inhibition was only initiated by doses of antigen which for adult guinea pigs were between 1—5 mg, while smaller doses had little or no effect. Asherson and Stone (1965) were of the opinion that the injection of soluble antigen affects immunologically competent cells directly and alters their subsequent response to the same antigen in Freund's adjuvant. Rowley and Fitch (1965) showed that antigenic stimulation induces an incidence of committed cells with high mitotic activity, which are highly susceptible to suppression after further contact with antigen. They used this hypothesis to explain why a schedule of repeated injections of antigen is the most effective for the induction of tolerance. Similarly, Nakano and Braun (1967) suggested that a second contact with antigen may lead to the destruction of stimulated cells. Dvorak and Flax (1966) suggested that some reactive cells derived from stem cells might be inhibited by relatively small amounts of antigen at an early, vulnerable stage of development. In a heterogeneous cell population a given concentration of antigen could simultaneously lead to inhibition of response in one type of cells and to sensitization of another. Since it is known that the selective induction of delayed hypersensitivity, without induction of antibody formation is dependent on the administration of a very small dose of antigen (Salvin, 1958; Feingold et al., 1964), it is not surprising that delayed hypersensitivity can also be inhibited by a relatively small dose. Similarly, both induction and inhibition of γM antibody synthesis requires a smaller dose of antigen than is needed for the γG antibody response. We assume, that various types of immune deviation, like selective inhibition of delayed hypersensitivity or supression of one type of antibody synthesis with concurrent stimulation of other types may have essentially similar mechanisms.

7. Ontogenetic Aspects

Studies of the antigen dose — immune response relationship during ontogenetic development revealed a number of complex situations. Most of the

experimental results indicate that immunologically competent cells are heterogeneous with regard to their sensitivity to a given antigen concentration and the molecular type of antibodies which they then produce. Young (1—3 month old) chickens form both γM and γG antibodies against HSA, but the dose of antigen required for the induction of *both* types is about 2 to 3 orders higher than for adult birds (VALENTOVA et al., 1967). It is possible that the antigen sensitive cells for IgM are differentiated sooner than precursors of cells producing after immunization γG antibodies. Another possible explanation would be that with advancing age a change occurs in the reactivity of a single line of differentiating cells, resulting in a state in which they are preferentially stimulated to IgG synthesis (reviewed by ŠTERZL and SILVERSTEIN).

An unusual pattern of immune response in relation to the antigen dose was observed in newborn rabbits by BOREL et al. (1964). The injection of large amounts of human RBC produced persistent IgM memory, while a small dose produced only a IgG anamnestic response. This was unexpected, since in rabbits given a large dose of antigen IgM memory was preceded by γG antibody formation.

In thymectomized mice, which resemble in some respects newborns, a 100 fold increase in antigen dose needed to elicit a primary hemolysin response to sheep RBC and a 10-fold increase in antigen dose needed to elicit "priming" was found (SINCLAIR and ELLIOTT, 1968).

It was demonstrated in newborn rabbits that the onset of the immune response was shortened and/or that antibody synthesis was higher if large amounts of particulate antigens were used (ŠTERZL and TRNKA, 1957; ŘIHA, 1961; BELLANTI et al., 1963; HAJEK and MANDEL, 1966). The ratio of the number of immunologically competent cells to the amount of antigen might be decisive for the final effect, but their interaction includes processes probably more complex then a simple quantitative relationship. The induction of tolerance can be prevented by an increase in the number of lymphoid cells in the organism (COHEN and THORBECKE, 1963), while on the other hand the decisive factor ought to be *only* the antigen concentration, assuming distribution of the cells to be uniform (SERCARZ and COONS, 1963). The probability of contact of the competent cell with the antigen molecule (ŠTERZL and JILEK, 1967) might perhaps apply in the case of particulate antigens in the very low dose range and might also provide an explanation for the higher threshold immunizing dose needed in the immature animal which has a smaller number of competent cells than adult animals. However, in the case of soluble antigens, their molar concentration in the tissues is so high, that a uniform access to immunologically competent cells can be assumed.

The neonatal period is particularly suitable for induction of skin graft tolerance (review by HAŠEK et al., 1961). However, comparison of the dose involved with such an antigen capable of cell division is difficult. In the adult organism, foreign cells are destroyed by the host's immune reaction, while in the immunologically immature animal they are able to repopulate and thus actually form a massive source of antigen in further development. Data on

antigen dose requirements for the induction of tolerance to non-replicating antigens are at variance, however. The early studies — most of which were carried out in rabbits, using protein antigens — all showed better tolerance in animals induced at newborn age (Smith, 1961). However, in these experiments newborns were compared with animals a few weeks old, which again differ from adults, as will be shown below. Comparative studies with centrifuged BGG (Dresser, 1962) and pneumococcal polysaccharide (Siskind et al., 1963) in mice demonstrated that the tolerance-inducing dose of antigen appeared to be about the same in the neonatal period and at later age. Wolfe (1962) actually has observed that tolerance to BSA induced in newly hatched chickens was more labile than tolerance induced in adult birds.

Study of the kinetics of loss of tolerance at different ages revealed differences between adult and juvenile (intensively growing) animals. Tolerance induced in newly hatched chickens to allogeneic RBC (Mitchison, 1962b) or in ducklings to xenogeneic RBC (Hašek, 1963) disappeared more slowly in adult birds than in juvenile birds. The length of time for which tolerance persisted after the antigen had been eliminated or removed by passively administered antiserum increased with age. For some time after tolerance has disappeared in ducks, it can be reinduced by an antigen dose which produces immunity in the controls (Hašek and Puza, 1962; Humphrey, 1964a). This "reminiscence of tolerance" is largely dependent on the age of the animal, being of considerably longer duration in adult individuals (Hašek, 1963). However, because of the need for repeated injections of antigen for the induction of tolerance to RBC, birds tested in adult life were also pre-exposed to larger doses of antigen before. In experiments in which tolerance to HSA was induced by the *same* dose of antigen in adult and juvenile birds, it was found more difficult to induce tolerance in juvenile than in adult individuals (Iványi et al., 1964a). Mitchison (1965) likewise found that tolerance induced to the same dose of BSA in mice of different ages led to slower recovery in older animals.

The capacity for immune response increases with age up to adult life (Wolfe et al., 1957; Makinodan and Peterson, 1962, 1964), while the age- relationship of inducibility and stability of tolerance is evidently more complex. It seems possible to explain this on the basis of 2 main factors whose development is illustrated in diagrammatic form in Fig. 8. The curve X represents the actual immunological potential of the animal, which increases with age; this factor expresses the size of the initial dose of antigen required for the induction of tolerance. The curve $S—X$ represents the rate of differentiation of antigen-reactive cells from stem cells and expresses the organism's antigen requirements for maintenance of tolerance. It is apparent from Fig. 8, that the high rate of cellular replacement ($S-X$) and an already well developed number of antigen-reactive cells (X) in the juvenile age might explain why is this period the less convenient for immunological tolerance.

Lastly, there is another factor which may be of significance in ontogenetic studies. It was found that the "space" of distribution and half-life of de-

gradation of protein antigens is dependent on the age of animal (IVÁNYI et al., 1964b). Because of greater dilution in the tissue fluids and rapid catabolism, antigen concentration can fall faster in juvenile animals than in adult ones to below the critical level necessary for induction and maintenance of immunological tolerance.

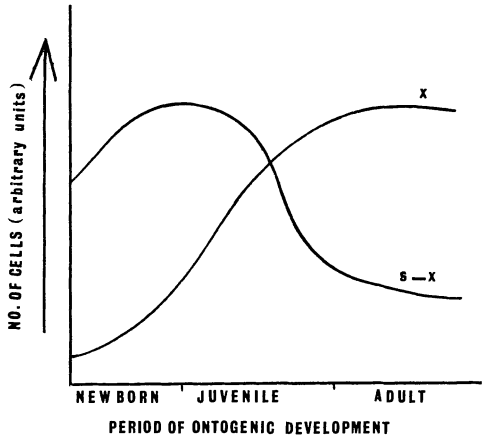

Fig. 8. Hypothetical curves expressing the number of antigen-reactive cells (X) and the rate of their differentiation from stems cells ($S-X$) in various periods of antogenic development

8. Treatment with X-rays and Drugs

a) X-irradiation

The effect of the dose of antigen in X-irradiated animals was first pointed out by TALIAFERRO and TALIAFERRO (1954), who found that small amounts of sheep RBC resulted in rabbits in a higher degree of suppression of haemolysin synthesis than larger doses of antigen. Similarly RITTENBERG and NELSON (1963) have found in irradiated rabbits the antibody response to 10 mg BSA completely suppressed, while the response to 100 mg HSA was only delayed. It was assumed that antigen persisting for a prolonged length of time could sensitize cells which had already recovered from X-irradiation. However, if still larger doses of antigen are injected, tolerance of longer duration than in unirradiated controls can be induced in sublethally irradiated animals (DIXON and MAURER, 1955). Attribution of this effect simply to a decrease in the number of immunologically competent cells is not altogether satisfactory, as there are grounds for assuming that the antigen concentration in the extracellular fluid remains the same in both the treated and control animals, i.e. that a single cell is acted upon by the same concentration of antigen molecules. The possibility of a change in the sensitivity of irradiated cells to antigenic stimulation was considered by HUMPHREY (1962) and NACHTIGAL and FELDMAN (1963). NETTESHEIM et al. (1967) using a syngeneic transfer system has compared the effect of the concentration of antigen on the stimulation of untreated and irradiated primed spleen cells. The minimum concentration

necessary for initiation of a near maximum antibody response was about 10^5 times greater for irradiated than for unirradiated spleen cells.

Apart from its effect on the actual population of antigen reactive cells, X-irradiation may activate the formation of new cells from stem cells. A raised threshold of stimulation of isolated primed cells is thus not at variance with the enhancing effect of X-irradiation in the induction of tolerance. It can be assumed that the irradiated animal can in many respects be regarded as similar to the early phase of ontogenesis, particularly in relation to the evidence that the antigen dose required for induction of immunity is higher than in normal adult individuals, while the dose needed to induce tolerance is lower.

b) 6-Mercaptopurine

Despite the fact that there are numerous immunosuppressive drugs, the significance of the dose of antigen has been evaluated only for 6-mercaptopurine (6-MP). Nathan et al. (1961) and Schwartz and Dameshek (1963) showed that 6-MP-induced suppression of antibody formation was proportional to the dose of antigen administered. A possible explanation of this finding is that γG antibody synthesis may be more susceptible to 6-MP than γM antibody formation (Sahiar and Schwartz, 1965), as larger doses of antigen are necessary for the induction of γG antibody formation (section 3a). This might also partly explain the absence of antigen dose dependence of the action of 6-MP on the immune response of rabbits to S. typhosa endotoxin, which elicits only γM antibody synthesis.

Inhibition of γG antibody formation occurs if 6-MP is administered together with antigen and on subsequent days after immunization. However, enhancement of γM antibody synthesis was demonstrated if a one week's course of 6-MP injections was given a few days before immunization by a small — almost sub-immunogenic dose of BGG (0.02 mg) to rabbits (Chanmougan and Schwartz, 1966). The authors explain their results as a consequence of the transformation of precursor lymphocytes to antigen-sensitive haemocytoblasts by nucleic acids or their degradation products released from cells injured by the action of this drug. Another conclusion of their results indicates that only part of the precursor cell population is activated in this manner, i.e. the part responsible for the IgM response.

9. Conclusions

Since hypotheses concerning the basic mechanisms of the immune reaction lack experimental verification in several respects, it is difficult to find interpretations, how the dose of antigen influences immunological reactions. However, a study of antigen dose — response relationships can make a decisive contribution to the understanding of some of these problems. In the following discussion we would like to draw attention to the processes by which studies of the antigen dose furnished significant informations.

1. Uptake of antigen by cells of the RES. Cells of the RES, mainly macrophages, remove antigen from the circulation at an exponential rate. The mechanism of uptake of antigen is non-specific because: a) changes of several orders of antigen concentration in the blood do not alter the rate of protein catabolism, but alter the immune response; b) the half-life of antigens is the same in tolerant and non-tolerant animals. Notwithstanding, it is possible that the participation of macrophages is required in the immune response to some antigens.

2. Interaction of antigen with antigen-sensitive cells. We presume the existence of receptors on antigen-sensitive cells. The quantitative conditions of interaction between antigen molecules with the receptor could (at least to some extent) be a cause of the heterogeneity of the response. The class of antibody is not clearly correlated with the length of the differentiation process, as it is in the case of haemoglobin; this is also evidence for heterogeneity of the cell population, probably in terms of various receptor concentrations or with different induction and inhibition thresholds.

An alternative explanation would be, that specific antigenic determinants occur intracellularly in antigen-reactive cells. Their concentration would be of critical importance, whether the outcome of differentiation will be immunity or tolerance. This concept does not require the presence of antigen in cells in the phase of specific antibody synthesis.

Interaction of an antigen with the cell receptor triggers cell differentiation and proliferation. The rate of these processes is dependent on antigen concentration. Some of the differentiating cells produce antibodies, but others do not reach this phase of specific synthesis; their product is not specific to the antigen and yet they increase in number with the size of the antigen dose.

3. The effect of antigen on stem cells. The immune response is not only the outcome of the response of the antigen-reactive cells actually present before introduction of antigen. An important part is also played by the interaction of antigen with cells newly differentiated from stem cells. The rate of this differentiation of new cells is influenced by the "nonspecific" effect of antigen, which could be determined by its physical properties (adjuvancy) or secondarily, through the release of stimulant substances from disintegrating cells. The differentiation of stem cells is activated especially after large doses of antigen, in early ontogenesis and under the influence of certain drugs and X-irradiation. The newly differentiated cells appear to have a higher stimulation threshold and a lower inhibition threshold, in relation to the antigen concentration. Stormy differentiation after large doses of antigen does not allow the expression of specific function in the cells, and in this respect the histological picture of antigen overloading can be regarded as the outcome of a compensatory reaction of the stem cells, which is ineffective, however.

4. The class of antibodies; immunological memory. The synthesis of γM and γG antibodies differ in respect of antigen dose required for induction and inhibition. However, at the present time there is still no decisive evidence, whether the competent cells have a predisposition in respect of heavy chain

synthesis or whether there is a "switch over", which would be regulated by the concentration of antigen. The dose of antigen determines in some degree the differentiation of competent cells either into antibody producing cells or to cells containing the specific information in a latent form (memory). Dose-response studies indicate that separate cell lines responsible for the secondary γM and γG antibody formation, with different cytokinetics exist. As with antibody-producing cells, the rapid disappearance of IgM memory is evidence of a suicidal type of differentiation. The gradual development of IgG memory in vivo may be evidence for proliferation of the originally committed cells.

5. Immunological tolerance. Inhibition of a single cell can be the outcome of interaction with a supraoptimal antigen dose. The threshold inhibitory concentration may vary with the antigen and the type of cell. Tolerance at the whole organism level requires inhibition of new cells differentiating from stem cells. The kinetics of cell-replacement determines the rate of escape from immunological tolerance. Persisting antigen can prevent the differentiation of new cells or continually inhibit them by some unknown mechanism.

6. Effect of small doses of antigen. Delayed hypersensitivity (irrespective of effector mechanisms) could be interpreted as a reaction of cells which can be induced by very low amount of antigen in the presence of strong nonspecific stimulation. In the absence of an adjuvant factor, a small dose of antigen inhibits these cells, leaving those responsible for humoral antibody production with a higher stimulation threshold capable of reaction. In the case of weak antigens, and especially of antigens with poor adjuvancy, inhibition can also extend to humoral antibodies. However complete inhibition is limited to a restricted type of antigens and in most systems small doses inhibit only one type of reaction, while another reactions take place (devivation).

7. Ontogenesis. The difference between the immune reactivity of the adult and immature organism should take in account several factors. In comparison with adult animals, an individual in the "adaptive" preiod is characterized by: a) a smaller number of antigen-reactive cells; b) higher rate of differentiation of stem cells into antigen-reactive cells; c) a higher antigen dose threshold needed for immunization; d) predominance of IgM synthesis; e) larger "space" of distribution and a higher rate of catabolism of protein antigens.

The following abbreviations were used:

RBC = red blood cells, HSA = human serum albumin, BSA = bovine serum albumin, BGG = bovine gamma globulin, KLH = keyhole limpet haemocyanin.

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Structure and Functions of Mesosomes of Gram Positive Bacteria

Antoinette Ryter

With 21 Figures

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Introduction

The most striking features which appeared on sections when bacteria were examined for the first time with the electron microscope fifteen years ago, were the high electron density of the cytoplasm and the lack of organelles. Thus, the usual structures and organelles apparent in thin sections of higher cells such as nuclear membrane, mitotic apparatus, mitochondria, endoplasmic reticulum, and golgi apparatus seemed to be absent in bacteria, although it was obvious they must have an organized electron transport system and a mechanism for ensuring equipartition of genetic material during division.

With the improvement of fixation and embedding techniques membranous structures were observed about ten years ago in *Mycobacterium avium* (Shinohara et al., 1957), in *B. subtilis* (Ryter and Kellenberger, 1958), and in Streptomyces (Glauert and Hopwood, 1959). Since then, they have been found in many genera of Gram positive bacteria (Kellenberger and Ryter, 1964; Gel'man et al., 1967; Salton, 1967). They were termed "chondrioid" (van Iterson and Leene, 1964), "intracytoplasmic membrane" (Glauert and Hopwood, 1961), "plasmalemmosome" (Edwards and Gordon, 1962) and "mesosome" (Fitz-James, 1960). The name mesosome has received wide acceptance in the literature and is now usually employed.

Mesosomes of Gram positive bacteria show different and rather complex configurations in section. Most commonly, they appear as a pocket formed by the cytoplasmic membrane filled with vesicles, tubules or lamellae. Their

number and size varies with the species, but generally they are rather numerous and well developed.

By contrast, in most Gram negative bacteria grown under normal conditions, membranous structures are seldom observed and are inconspicuous (VAN ITERSON, 1962; GEL'MAN et al., 1967; RYTER, 1968; COTA-ROBLES, 1966). Their appearance differs from that of mesosomes in Gram positive in that they present no tubules or vesicles but seem instead to be composed of folded or interdigitated membranes.

Recent observations have shown that membranous structures can attain greater prominence in certain strains of *E. coli* (SCHNAITMAN et GREENWALT, 1966; KOHIYAMA et al., 1966) or under certain growth conditions, e.g. when *E. coli* cells are held at 40° (STEED and MURRAY, 1966). POINDEXTER, STOVE and COHEN-BAZIRE (1966) have also reported the presence of large and rather complex structures in *Caulobacter crescentus* which present an aspect and a location similar to mesosomes of Gram positive bacteria.

These different membranous structures must be clearly distinguished from the extensive intracellular membranous systems found in nitrogen-fixing, nitrifying and photosynthetic bacteria which are related to the specific functions of these organisms (HICKMAN and FRENKEL, 1959; 1965; DREWS and GIESBRECHT, 1965; GIBBS et al., 1965; REMSEN et al., 1967; COHEN-BAZIRE, 1963; MURRAY and WATSON, 1965; REMSEN et al., 1968; ALLEN, 1968).

At present, information concerning mesosomes of Gram negative bacteria is very limited and it is not yet known whether they have analogous functions to those of mesosomes of Gram positive bacteria. For these reasons, we will confine our review to mesosomes of Gram positive bacteria whose structure, behaviour, functions and relationships with other cellular constituents have been studied intensively in recent years.

Structure

Variations of a hypothesis of mesosome structure have been proposed (FITZ-JAMES, 1960; KOIKE and TAKEYA, 1961; IMADEA and OGURA, 1963) in which the mesosome is represented as a pocket made by a large invagination of the plasma membrane. This invagination was thought to form later lamellar, tubular or vesicular extensions which were coiled into the pocket. These hypotheses do not account very well for the presence of the very large number of vesicles which are observed in many mesosomes, particularly after double fixation in glutaraldehyde-osmium (Fig. 5). The study of protoplast formation in some species of bacillus (FITZ-JAMES, 1964; RYTER and JACOB, 1964; RYTER and LANDMAN, 1964; WEIBULL, 1965) and in *Listeria monocytogenes* (GHOSH and MURRAY, 1967) shed new light on this subject. It was shown using the technique of thin sections that, when bacteria are introduced into a hypertonic medium, the mesosomal tubules are expelled from the cytoplasm and accumulate between the membrane and the wall. The digestion of the wall by lysozyme, which leads to protoplast formation, releases these tubules into the medium.

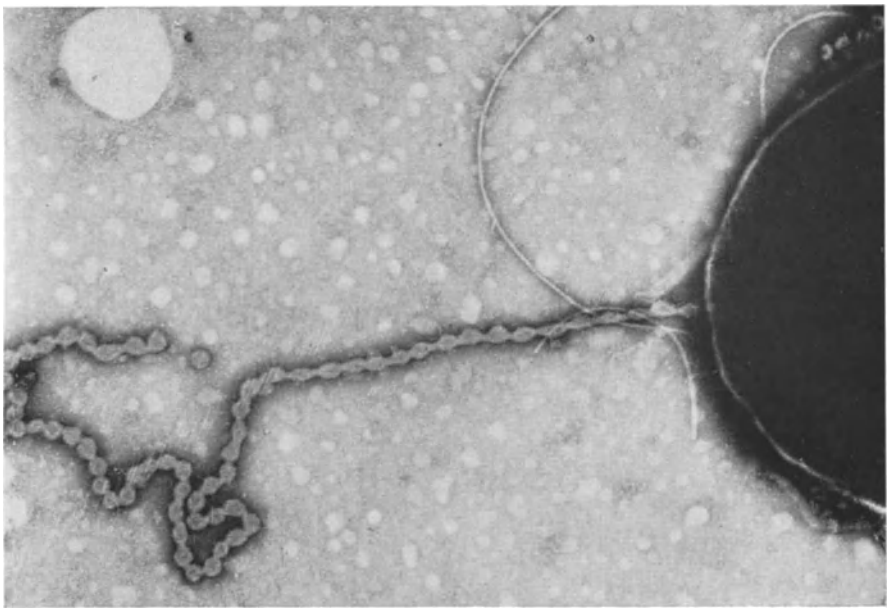

Fig. 1

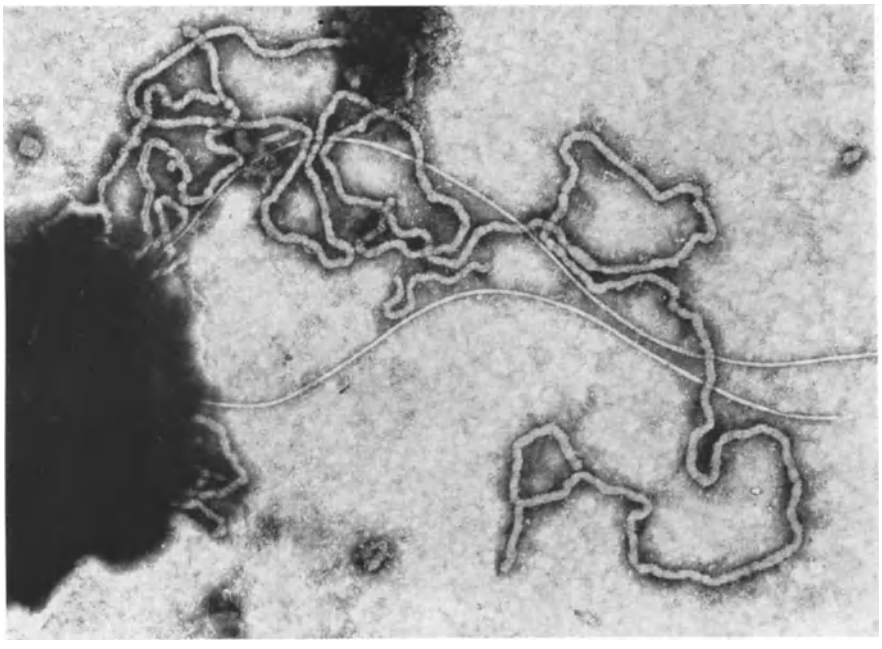

Fig. 2

Fig. 1 and 2. Negatively stained protoplasts to which are attached extruded mesosomal tubes. 33,000 × and 28,000 ×

In negative staining preparations, mesosomal tubules of *B. subtilis* (RYTER et al., 1967), (Figs. 1 and 2) and *B. megaterium* (RYTER, 1968) appear as long appendages which generally remain attached by one end to the protoplast

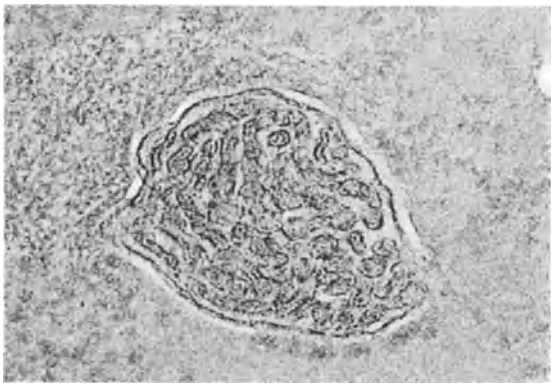

Fig. 3. Section of mesosome of *B. subtilis* in which the string of beads structure is well visible. 123,000 ×

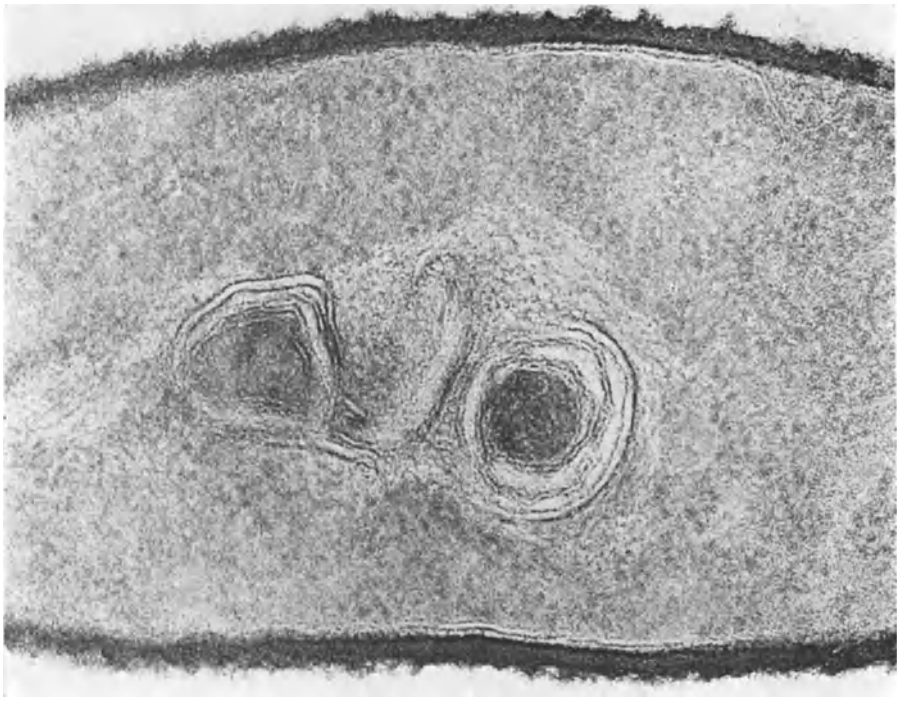

Fig. 4. Mesosome of *B. subtilis* in the nuclear region. Osmium fixation. 105,000 ×

surface. Their length varies very much, some of them can reach 20 μ. They consist of a chain of small vesicles (500 to 700 A in diameter in *B. subtilis* and about 1000 A in *B. megaterium*), and thus look like a string of beads. In some places, this structure is interrupted by straight segments (Fig. 6). These appendages are very fragile and the pearl string structure is easily modified during the negative staining procedures (Fig. 2). However, the string of beads structure is certainly not an artefact of this technique since it is also observed in shadowed preparations (RYTER, 1968). Even in thin sections, the

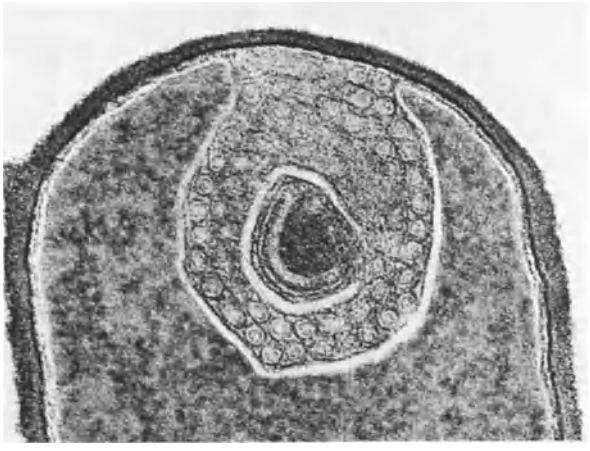

Fig. 5. Mesosome of *B. subtilis* constituted with a lot of vesicles. Glutaraldehyde-osmium fixation. 95,000 ×

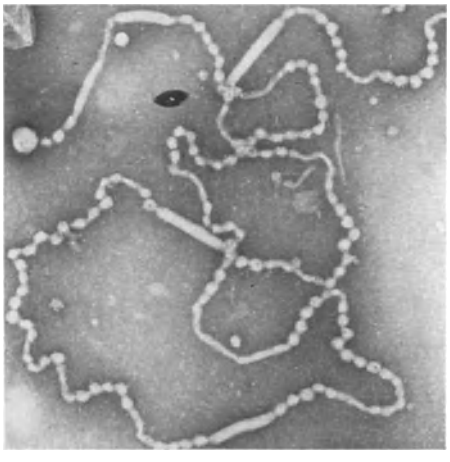

Fig. 6. Mesosomal tube in negative staining showing the string of beads structure interrupted by straight segments. 23,000 ×

string of beads structure can be detected when the angle of section is favorable, either inside the mesosome pocket (Fig. 3) or in plasmolysed bacteria or near protoplasts (RYTER, 1968). The large number of vesicles found in mesosomes in freeze-etching preparations also confirms these results (REMSEN, 1968; NANNINGA, 1968).

The quality of fixation considerably influences the aspect of mesosomes. Osmium fixation very frequently seems to transform the pearl string structure into a tubular structure. This explains the lamellar cross-section of mesosomes commonly observed after this type of fixation (Fig. 4). Glutaraldehyde fixation preserves the vesicular shape better. When bacteria have been fixed by the double glutaraldehyde-osmium procedure, most of the mesosomes contain a lot of vesicles and few lamellae or longitudinally-cut tubules (Fig. 5). The tubules or lamellae probably correspond to the straight segments found in the

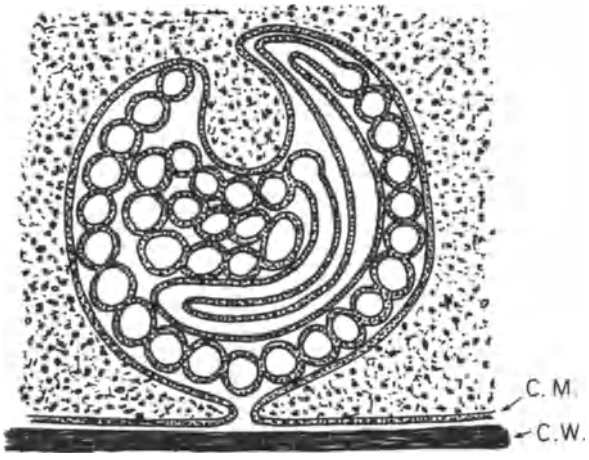

Fig. 7. Representation of a mesosome. *CM* cytoplasmic membrane; *CW* cell wall

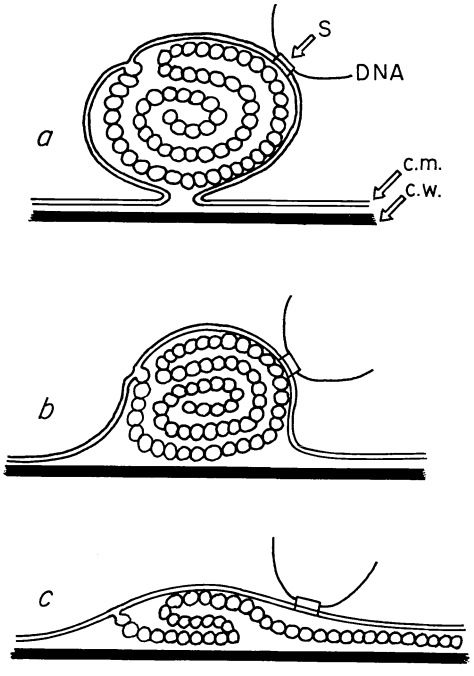

Fig. 8. Representation of the process of mesosomal extrusion when the cell is introduced into a hypertonic medium. *CM* cytoplasmic membrane; *CW* cell wall; *S* attachment structure connecting the DNA and the membrane

uncoiled mesosomes (Fig. 6). The spherical bodies containing cytoplasmic material which are sometimes observed in mesosomes probably correspond to a large invagination of the mesosomal membrane (Fig. 7).

In summary, the mesosome of Bacilli can be represented by Fig. 7. The mesosome pocket is formed by a membrane invagination as FITZ-JAMES (1960), IMADEA and OGURA (1961) have already proposed. Inside this invagination,

there is probably only a single coiled tube. When the bacteria are introduced into a hypertonic medium, this pocket opens and flattens out and the tube is pushed out into the space between membrane and wall (Fig. 8).

This structure, deduced essentially from observations made on strains of the species Bacillus can be roughly applied to other Gram-positive bacteria. Some micrographs of Streptomyces (GLAUERT, HOPWOOD, 1961), of Actinomyces (BLECKING et al., 1964) and even of a photosynthetic bacterium (PFENNIG and COHEN-BAZYRE, 1967) clearly show beads-on-a-string structures. Some differences exist from one species to another probably in the size and the number of vesicles but the variations in mesosome morphology among bacterial species which seem to be documented in the literature (see GEL'MAN et al., 1967) is probably due to the quality of fixation. Depending on whether the

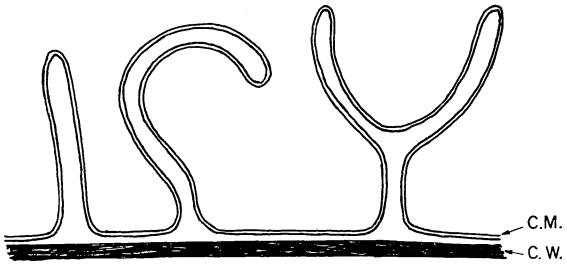

Fig. 9. Representation of different kinds of membrane invagination. *CM* cytoplasmic membrane; *CW* cell wall

vesicular structure has been conserved or not, mesosomes will seem to consist essentially of lamellae or tubes.

An exception perhaps exists in *Steptococcus pyogenes* in which the expulsion of mesosomes during protoplast formation is not complete (COLE, personal communication). It is not yet known whether this behaviour is due to a difference in mesosome conformation or to a more rigid membrane. OP DEN KAMPF et al., (1967) observed in *B. megatherium* that the rigidity of the membrane is increased at low pH and that in this case the mesosomes are not expelled from the cytoplasm during protoplast formation.

Besides normal mesosomes, some bacteria contain membranous structures which look like simple membrane invagination without vesicles or tubules (TOMASZ et al., 1964; EDWARDS and STEVENS, 1963). These probably correspond to mesosomal pockets which have collapsed in different ways (Fig. 9) and which lack mesosomal tubules.

No information has yet been obtained on the insertion point of the mesosome tube. Since the mesosomal tube exhibits the triple-layered profile characteristic of the unit membrane in thin sections, it is probably formed by an invagination of the cytoplasmic membrane in a region of the mesosomal pocket. It is also not yet established whether vesicles contain anything and what type of metabolic interchange occurs between the inside of the tube and the cytoplasm. The fact that vesicles separate from one another and easily burst when mesosomal tubes are introduced into a hypotonic medium, indicates

that the inner osmotic pressure is relatively high (FERRANDES et al., in preparation).

As shown by three-dimensional reconstructions of growing cells of *B. subtilis* made with the help of serial sections (RYTER and JACOB, 1964; FUHS, 1965), mesosomes are always connected to the cytoplasmic membrane (Fig. 18). That means that the pockets of all mesosomes are more or less opened outwards. The free penetration of phosphotungstate into the mesosomes and membrane invaginations suggests that there are no permeability barriers external to these structures (ZWILLENBERG, 1964; BLADEN et al., 1964).

Behaviour of Mesosomes

As has already been noted, the number and the size of mesosomes varies with the species. Within the cells, they are found usually in the nuclear region and at the septa. In species which possess many mesosomes per cell, these organelles are also located along the cytoplasmic membrane (see GEL'MAN et al., 1967).

No study seems to have been published about the influence of physiological conditions on the mesosomes. From observations made in my laboratory on *B. subtilis*, the number and the size of mesosomes do not change very much with the growth medium and when some differences do appear, no correlation can be established between the growth rate and the size of the number of mesosomes (FREHEL, to be published). Observations made by CONTI et al. (1968) on *Staphylococcus epidermis* and on *B. macerans*, both facultative aerobic strains, showed that mesosomes exhibit the same size, number and structure regardless of whether these bacteria are grown under aerobic or anaerobic conditions.

By contrast, in the case of sporulation, mesosomes increase in size and decrease in number just before the beginning of sporulation in many strains of *Bacillus* (RYTER and JACOB, 1964; FITZ-JAMES, 1960). Three-dimensional reconstructions of many *B. subtilus* cells made with the help of serial sections show that only two big mesosomes are present in the first stage of sporulation. One participates in the formation of the septum of sporulation (Fig. 10) and enters the prespore at stage III, whereas the other remains in the mother cell (RYTER and JACOB, 1964). The presence of two big mesosomes seems to be closely related to the sporulation process and not to the termination of growth, since all mutants which are blocked at stage 0 of sporulation (they present no morphologic sign of sporulation) contain numerous small mesosomes in each cell (RYTER et al., 1966). In this special case, therefore, physiological conditions determine a specific behaviour of mesosomes.

The mode of formation of mesosomes is still not well understood, but it is obvious that they are formed from the cytoplasmic membrane. KAKEFUDA et al. (1967) have observed that the membrane pocket of some mesosomes presents a normal triple layered structure, whereas in others the electron dense layer in contact with the cytoplasm is extremely thin. This latter type of mesosome is generally wide open outwards. The authors suggest that this

type of mesosome might by young mesosomes, whereas the former type, which are tightly closed, would correspond to old mesosomes.

FITZ-JAMES (1967—68) reported very interesting results concerning lipid synthesis. Short pulses of radioactive lipid precursors were given to *B. cereus* and, immediately afterwards, a mesosome fraction was separated from a cytoplasmic membrane fraction. A great part of the radioactivity was found in the mesosome fraction. Pulse-chase experiments showed that the radioactivity originally located in mesosomes moves into the membrane fraction. These results strongly suggest that the mesosomes might be the site of membrane

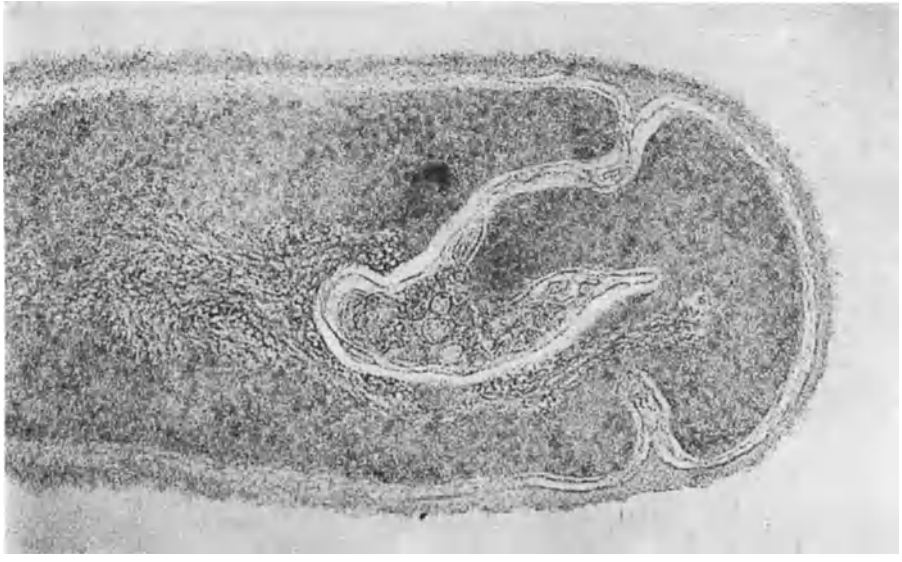

Fig. 10. *B. subtilis* cell at the beginning of sporulation. A big mesosome is connected to the septum of sporulation. A part of the nuclear material is entering the sporal part of the sporangium. 86,000 ×

synthesis. Such a hypothesis is very attractive because it fits very well with the fact that mesosomes are always present in the regions where obvious membrane growth occurs: at the septum of division or septum of sporulation. It seems, however, that other workers did not get the same results (PATCH, 1968; BURGER and LUBOCHINSKY, unpublished results). They found a similar distribution of lipid precursors in both fractions over the time period sampled.

Mesosomes are probably not stable organelles then, they can disappear in some cases. Indeed, as shown in Fig. 18, all nascent septa have mesosomes whereas many finished septa have none (RYTER and JACOB). These organelles are also easily altered under poor growth conditions without affecting the morphology of other cellular constituents. Mesosomes of *B. subtilis* are completely destroyed when these bacteria are left for 2 hours at 4° without aeration (Fig. 11). Starvation in phosphate buffer also changes the structure of mesosomes (FREHEL, not yet published) (Fig. 12). The tubules seem to disappear and the mesosomal pocket very often collapses completely and presents an

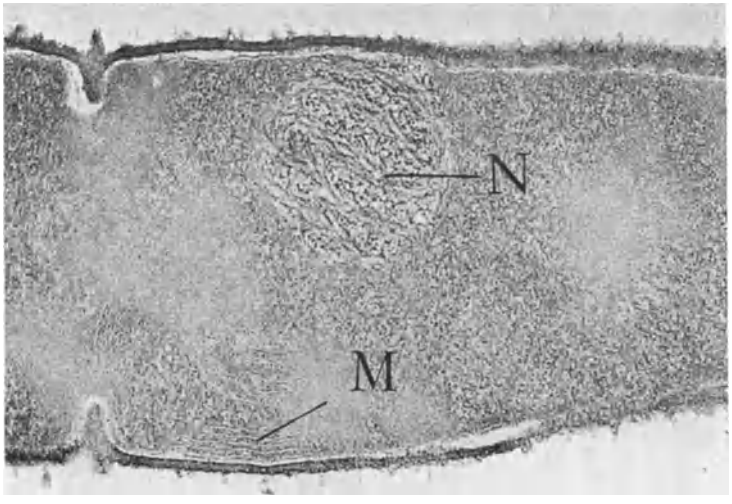

Fig. 11. *B. subtilis* cell after two hours at 4° without aeration. The mesosomes (*M*) have been destroyed and the nucleus is in contact with the cytoplasmic membrane. 63,000 ×

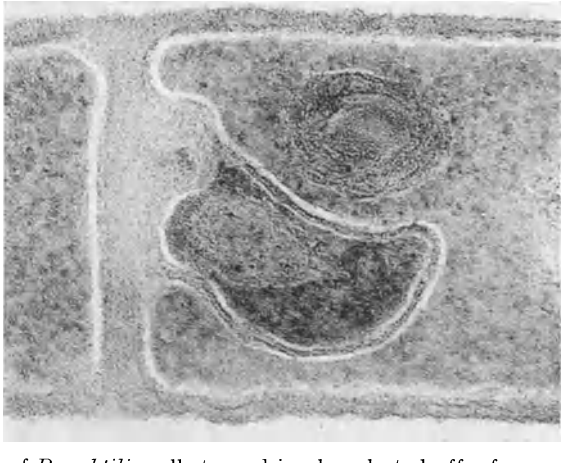

Fig. 12. Mesosomes of *B. subtilis* cell starved in phosphate buffer for one hour. Most of the vesicles have disappeared and the mesosomal pocket seems to have collapsed. 55,000 ×. (Micrograph made by C. FRÉHEL, Paris)

appeared close to that of the membranous invaginations observed in *Diplococcus pneumoniae* (TOMASZ et al., 1964). Similar alteration has been also observed in *B. thuringiensis* grown in the presence of m-tyrosine (an analog of tyrosine, ARONSON et al., 1967). The presence of chloramphenicol transforms the mesosomes into myelin-like structures (GIESBRECHT and RUSKA, 1968; and personal observation) (Fig. 13).

These diverse modifications indicate the extreme fragility and instability of mesosomes. It is probable that the cytoplasmic membrane is also affected by such factors as anaerobiosis and cold, which often produce lysis of *B. subtilis*.

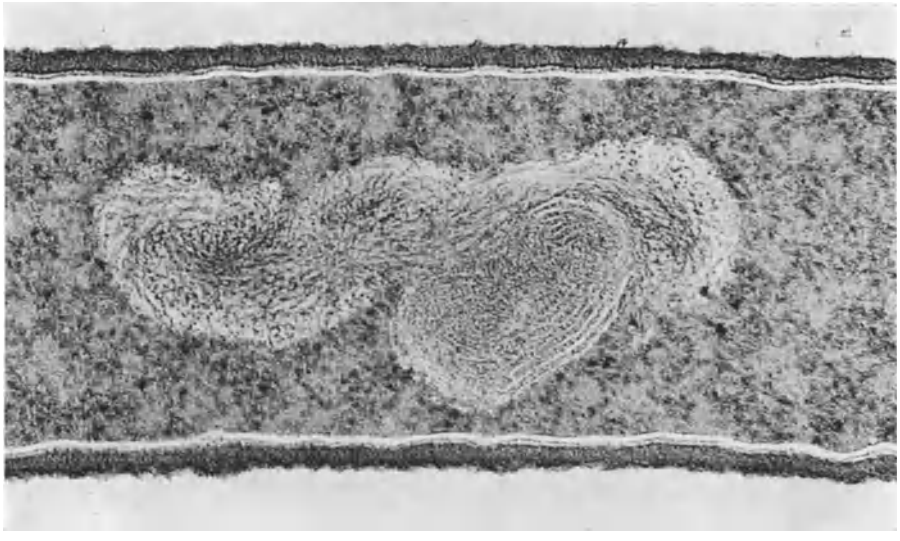

Fig. 13. *B. subtilis* cell treated for one hour with 10 γ/ml of chloramphenicol. The mesosome presents a particular lamellar structure. 80,000 ×

Functions

When mesosomes were first discovered, the question of their functions arose of course immediately. The fact that they were the first cytoplasmic organelles observed in bacteria explain why many different functions were attributed to them.

1. Their membranous origin and the presence of tubules suggested that they might be organelles homologous to mitochondria (GIESBRECHT, 1960; VAN ITERSON, 1961; GLAUERT and HOPWOOD, 1960; FITZ-JAMES, 1960).

2. Their localization along the membrane and at the septa suggested that they might play a role in wall synthesis (CHAPMAN and HILLIER, 1953; GLAUERT, 1962; OHYE and MURRELL, 1962; FITZ-JAMES, 1960; IMADEA and OGURA, 1963; VAN ITERSON, 1961; RYTER and LANDMAN, 1964).

3. Their frequent presence in the nuclear region indicated that they might be involved to some extent in nuclear function and division (GIESBRECHT, 1960; ROBINOW, 1963; VAN ITERSON, 1961; RYTER, 1963).

These questions have been intensively studied during the past few years and we shall examine whether all these functions can really be attributed to the mesosomes.

Respiratory Function

Before the discovery of mesosomes, biochemists and morphologists were in disagreement. The biochemists found respiratory activities in the cytoplasmic membrane; the morphologists, using mitochondrial staining procedures and tetrazolium salts, observed in the light microscope small granules localized in the cytoplasm along the membrane. The discovery of membranous structures in bacteria allowed everybody to agree again (MUDD et al., 1960).

The electron microscope studies, using potassium tellurite and different tetrazolium salts, confirmed the respiratory functions of mesosomes and provided more precise details (see GEL'MAN et al., 1967). Triphenyl tetrazolium is reduced in mesosomal regions (VANDERWINKEL and MURRAY, 1962; TAKAGI et al., 1965; TAKAGI et al., 1963; KAWATA and INOUE, 1965) and tetranitroblue tetrazolium (TNBT) forms dense precipitates in the meso-

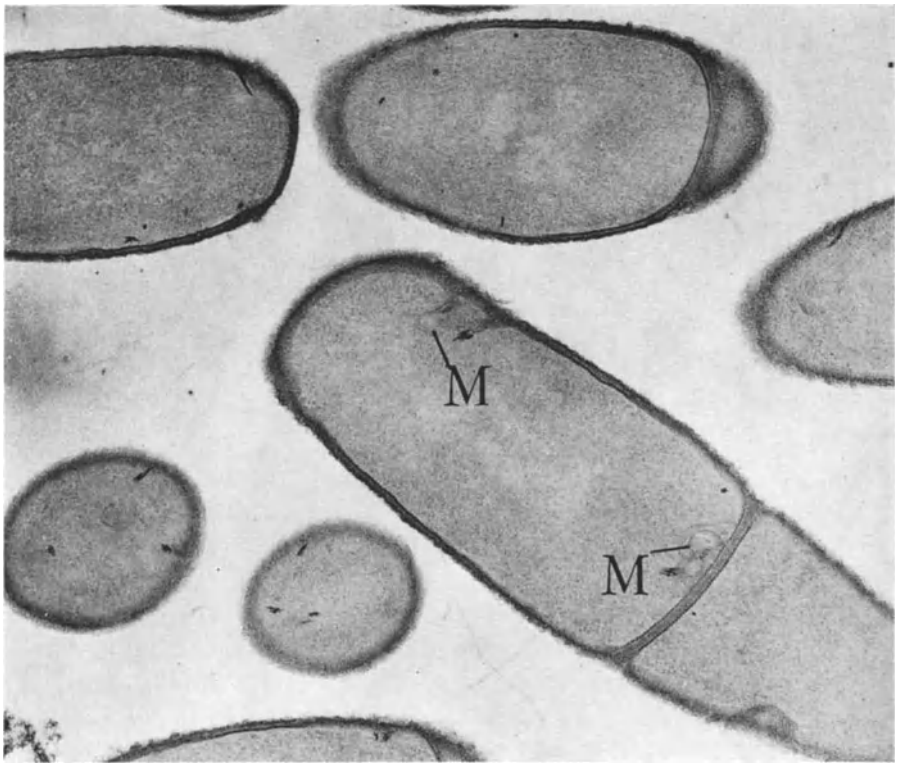

Fig. 14. *B. subtilis* cells treated for 30 minutes with 0.05% potassium tellurite. The tellurium needles deposit either on the cytoplasmic membrane or on the membrane which forms the mesosomal pocket, but never on the mesosomal tubules. 40,000 ×. (Micrograph made by C. FRÉHEL, Paris)

somes (SEDAR and BURDE, 1965; LEENE and VAN ITERSON, 1965; YAMAGUCHI et al., 1966). SEDAR and BURDE also observed dense deposits on some parts of the peripheral membrane and sometimes in the nuclear regions.

Potassium tellurite, which deposits in the form of dense needles on the mitochondrial cristae of higher cells (BARRNETT and PALADE, 1957), is observed in the same form along the bacterial membrane and sometimes in cytoplasmic regions close to the membrane (VAN ITERSON and LEENE, 1964; VAN ITERSON, 1965; RYTER, 1967; KAWATA and INOUE, 1965; TAKAGI et al., 1965; NERMUT, 1960). Tellurium needles were never observed in the mesosomes but VAN ITERSON and LEENE (1964) reported the presence of a dense, granular deposit on the mesosomal tubules.

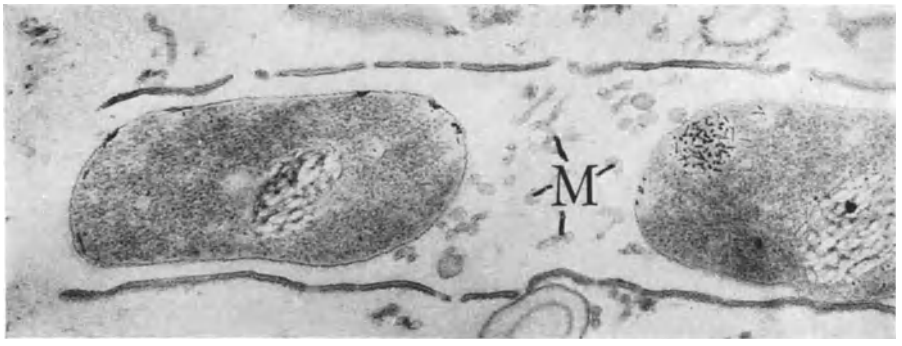

Fig. 15. *B. subtilis* cell in the course of protoplast formation treated with potassium tellurite. The tellurium needles are located on the cytoplasmic membrane but not on the extruded mesosomal tubules (*M*). A group of needles is visible in the cytoplasm of the right bacterium. 30,000 ×. (Micrograph made by C. FRÉHEL, Paris)

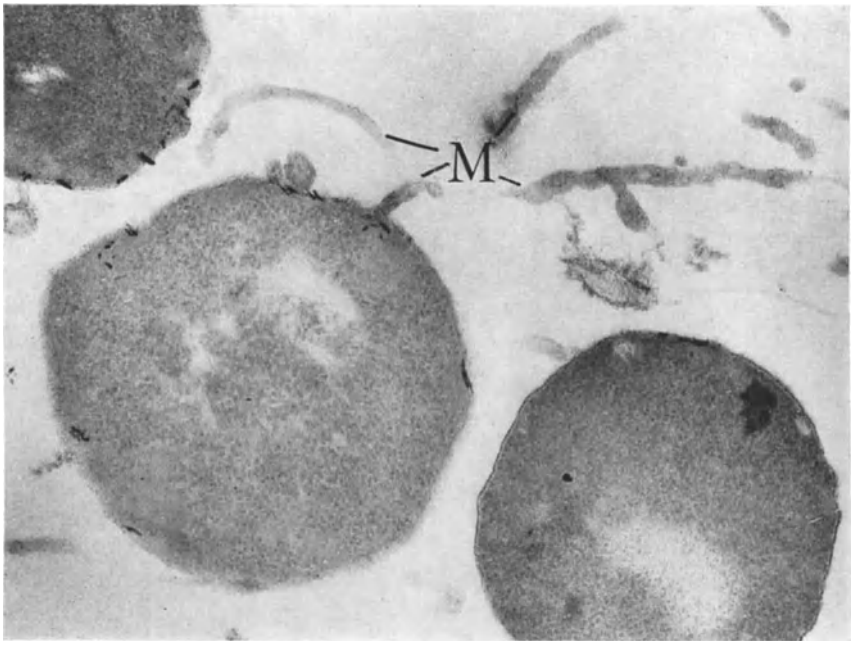

Fig. 16. Protoplasts of *B. subtilis* treated with potassium tellurite. The tellurium needles are located on the cytoplasmic membrane, very often close to the points of insertion of mesosomal tubes, but they are never found on the mesosomal tubes themselves (*M*). 42,000 ×. (Micrograph made by C. FRÉHEL, Paris)

We have recently made a careful study of potassium tellurite reduction in *B. subtilis* during growth and during protoplast formation (FREHEL et al., 1968; FERRANDES et al., in preparation). — It appeared that the needles are formed during the first thirty minutes of tellurite treatment. They are always located along the membrane and very frequently on the membrane which forms the mesosomal pocket (Fig. 14). In plasmolyzed cells and in protoplasts, they are found also on the membrane, most abundantly near the insertion

points of mesosome tubules (Figs. 15, 16). They were never observed either in intact mesosomes or on uncoiled mesosomal tubes.

The granular precipitate is found in the mesosomes only after one or two hours following the addition of tellurite. It is visible only after osmium fixation and not after glutaraldehyde fixation. It seems therefore that the granular accumulation on the mesosomes is not produced by the same reaction as that which leads to needle formation. Further, since bacteria are no longer viable 30 minutes after the addition of tellurite (personal communication), it is possible that the granular deposit is produced by unphysiological reactions.

It can be concluded that, in the case of potassium tellurite, the cytoplasmic membrane and especially the regions which constitute the mesosomal pocket display a redox reaction analogous to that which occurs on the mitochondrial cristae. By contrast, the mesosomal tubes do not appear to possess this type of function and therefore cannot be compared to mitochondrial cristae.

In summary, the cytochemical studies show that different cytochemical reagents are not reduced on the same membranous structures: triphenyl tetrazolium is apparently reduced only in the mesosomes, tellurite only on the membrane and tetranitroblue tetrazolium in both the mesosomes and the membrane. These results are not contradictory, since the reduction of these substances depends on their redox potential. According to observations made by NACHLAS et al. (1960), TNTB is reduced in the respiratory chain in the region of cytochromes. Triphenyl tetrazolium reacts probably after cytochrome C with cytochrome oxidase (VANDERWINKEL and MURRAY, 1962; NACHLAS et al., 1960). Judging from the redox potential of potassium tellurite at pH 7, this salt might receive electrons from the flavoprotein or quinones (FREHEL et al., 1968; FERRANDES et al., in preparation). The various reagents may thus reveal the presence of different enzymes of the respiratory chain.

The foregoing staining techniques cannot be controlled with precision and do not permit a clearcut enzymatic analysis of the membranous structures of bacteria. New techniques for the separation of mesosomal tubules from the cytoplasmic membrane probably provide a better basis for enzymatic studies. Introduced independently three years ago by FERRANDES et al. (1966) and FITZ-JAMES (1967—1968), they have been improved during the past year in various laboratories (ROGERS et al., 1967; REAVELY, 1968; RYTER et al., 1967; SARGENT et al., 1968) The mesosomal tubes are detached from the surface of the protoplasts, before or after centrifugation of the protoplasts. The lysis of the protoplasts provides the membrane fraction. The mesosomal tubes which remain in the supernatant are spun down at high speed and can be purified in sucrose gradients (FITZ-JAMES, 1967, 1968) or caesium chloride gradients (REAVELY, 1968).

The analysis of these fractions showed that most of the cytochromes are located in the mesosomal fraction (FERRANDES et al., 1966; FITZ-JAMES, 1968). These results fit well with the important reduction of triphenyl tetrazolium in the mesosomes and also with the increase in the number of mesosomes in *Caulobacter crescentus* grown in oxygen-limited culture and the abnormally high

content of heme pigment found in these bacteria (COHEN-BAZIRE et al., 1965). They are also in agreement with the fact that membranes of L-forms of Gram positive bacteria (which have no mesosomes) do not contain cytochromes (WEIBULL and GYLLANG, 1965). The localization of cytochromes must be studied further and more carefully, however, because it seems that, depending on the conditions under which protoplasts are formed, the cytochromes are detached from the membranes and then fractionate along with the mesosomal vesicles (BURGER and LUBOCHINSKY, personal communication). The localization of cytochromes in the mesosome vesicles may well be partly due to this artefact.

Further enzymatic analysis of mesosomes and membrane fractions was undertaken in various laboratories during the past year, but these results are not yet published. Some minor disagreements exist between laboratories. It seems, however, that several groups have found succinic-dehydrogenase in both the mesosome and the membrane fractions, whereas NADH dehydrogenase is found in large amounts in the membrane fraction and is nearly absent in the mesosome fraction (SARGENT et al., 1968; FREHEL et al., 1968; LUBOCHINSKY personal communication; ROGERS, personal communication). These preliminary results also indicate, as do the cytochemical observations that the mesosome fractions and the membrane fraction differ in their enzymatic content.

These conclusions are still preliminary and will have to be confirmed because the techniques of isolation of membrane fractions can produce many different artefacts, such as inactivation and detachment of enzymes. It has been observed, for example, that the introduction of bacteria into a hypertonic medium and the addition of lysozyme produce a decrease of cellular respiration (FERRANDES et al., in preparation). Moreover, the groups of tellurium needles which are found in plasmolyzed bacteria and in protoplasts treated with potassium tellurite (Fig. 16) (FREHEL et al., 1968; KAWATA et al., 1960) indicate that probably some enzymes are detached from the membrane, accumulate in the cytoplasm and are lost after the lysis of the protoplasts.

We can hope however that these difficulties will be overcome and that the respiration function of mesosomes will be clarified for the next years.

Role in Wall Synthesis

As has already been noted, in all the Gram-positive bacteria the mesosomes are very frequently associated with the septa of division. Further, in *B. subtilis*, three-dimensional reconstructions (RYTER and JACOB, 1964) showed that all nascent septa are in contact with one or two mesosomes, whereas completed septa do not necessarily possess a mesosome. These observations suggest that mesosomes participate in septum formation, possibly by providing cell wall precursors.

The participation of mesosomes in cell wall synthesis was also considered in conjunction with the study of protoplast multiplication. It has already been described how during their formation protoplast of Bacilli lose their mesosomes (RYTER and LANDMAN, 1964; FITZ-JAMES, 1964; GHOSH and

MURRAY, 1967). — The mesosomal tubes which may remain attached to their surface can apparently be completely eliminated after several washings (LANDMAN et al., 1968). These protoplasts are able to multiply in hypertonic soft agar medium (as L-forms) and retain some essential morphological characters of protoplasts: absence of wall and absence of mesosomes (RYTER and LANDMAN, 1964).

Since all the L-bodies descended from the inoculated protoplasts are also devoid of wall and mesosomes, it is evident that the transient lysozyme treatment has triggered a loss of important cell attributes which is transmitted through successive cell generations. It was postulated that the absence of mesosomes might be the cause of this incapacity to manufacture cell wall which persists as long as protoplasts grow on soft agar media but disappears when protoplasts are introduced into hard media containing for example 25—30% gelatin (LANDMAN et al., 1968). This process of protoplast reversion offered the opportunity to see whether mesosomes were formed again and whether their appearance precedes or follows the formation of the new cell wall.

Protoplasts of *B. subtilis*, carefully washed in order to eliminate all mesosome fragments, were introduced into gelatin medium. Samples were taken at different times and prepared for electron microscopy. The reversion process begins about 6 hours after suspension in gelatin medium. Its first morphological sign is the presence of a thin layer surrounding the cytoplasmic membrane, generally around the whole surface of the protoplasts. As it thickens, the new cell wall exhibits a fairly uniform thickness and when it finally reaches its ultimate thickness, it looks just like ordinary *B. subtilis* cell wall (Fig. 17).

The shapes and sizes of reverting cells are extremely variable. The branched forms which are often seen are undoubtedly cells in the process of division. Apparently, elongation can occur in several directions. The typical shapes of the bacillary form appear rather late, which indicates that the controls determining the characteristic rod shape comes into play only in later cell divisions and well after reversion has taken place.

Mesosomes are observed only very rarely. A quantitative study of the number of mesosomes per cell shows that about 3/4 of the reverting cells have no visible mesosome at all. The rare mesosomes which form are found quite early in reversion as well as later, but never in protoplasts not yet engaged in the reversion process. This observation indicates that they cannot exist in the absence of wall. Since mesosomes seem to consist of corrugated membrane tubes rolled up inside the mesosomal pocket, it is plausible to assume that a rigid wall casing is required both for the formation and the maintenance of such structures. Similar considerations apply to septum formation in protoplasts and L-bodies where the division process is considerably disturbed as soon as the wall is absent.

The fact that mesosomes appear after the first layer of wall and that many reverting cells are completely devoid of mesosomes suggests that mesosomes do not play an essential role in wall synthesis.

It may be objected that fragments of original mesosomes might still adhere to the protoplast surface and that these mesosome residues participate in reversion. However, this possibility seems to be eliminated by the fact that L-bodies descended from the original protoplasts through many transfers, revert just as efficiently in gelatin as the freshly made protoplasts.

Further, extensive direct observations by shadowing and sectioning techniques of the protoplast inocula used in these experiments have not revealed the presence of any adherent mesosome fragments.

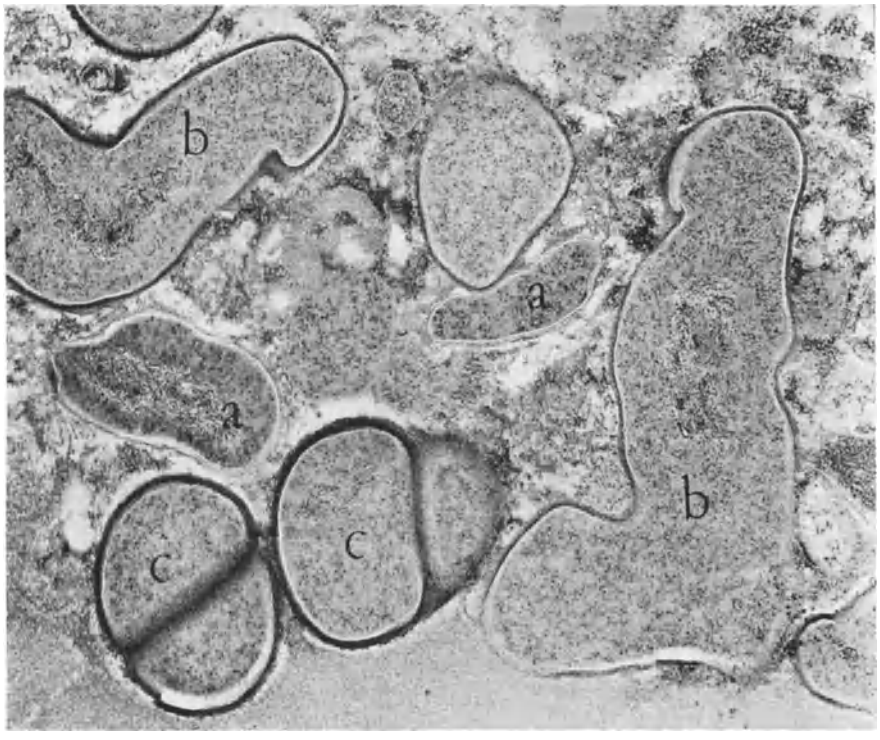

Fig. 17. *B. subtilis* protoplasts reverting to the bacillary form. Two protoplasts have just begun to manufacture a new cell wall (*a*); others are more engaged in the reversion process (*b*); two others (*c*) already possess a normal wall and are dividing. No mesosome can be seen in these cells

The physical state of the reversion medium plays an essential role in the reversion process. When gelatin tubes containing reverting protoplasts are heated for some minutes at 40° during the first four hours following the introduction of protoplasts into the reversion medium, the reversion is set back by as much as two hours. The physical state of the gelatin media does not favour the formation of mesosomes, as might be supposed, but perhaps allows a local accumulation of wall material which is not well affixed to the protoplast surface and which is swept away by the fluid motion at 40°.

The enzymatic analysis of mesosome and membrane fractions recently undertaken by BURGER (personal communication) complements the morpho-

logical results in showing that two enzymes involved in thecoic acid synthesis are located in both fractions, in different amounts. It can be concluded that mesosomes play a role in the wall synthesis but that this role is not specific to the mesosomes and that, in their absence, the membrane can take over this function.

Role in the Nuclear Function

The study of possible mesosome participation in nuclear function was stimulated by a hypothesis put forward by JACOB et al. (1963) according to which the bacterial nucleus is attached to the membrane. Three dimensional

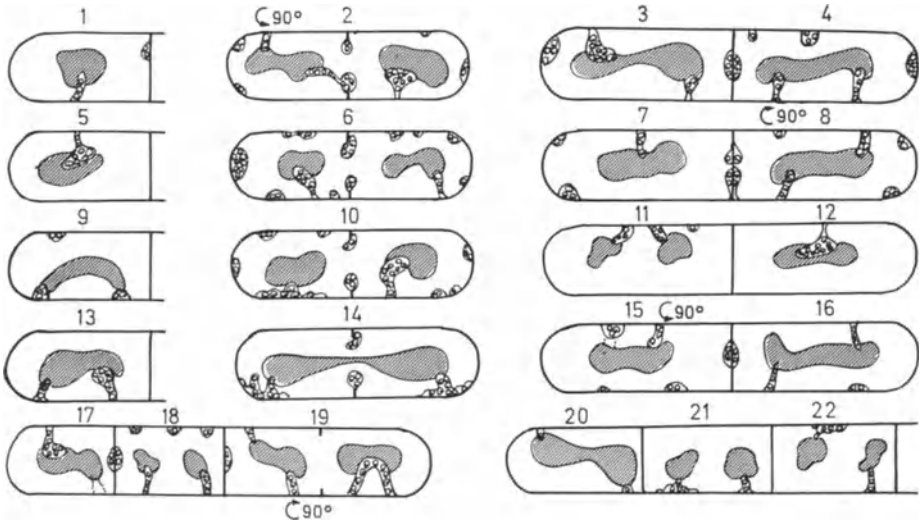

Fig. 18. Representation in two dimensions of the three-dimensional reconstructions obtained from serial sections of *B. subtilis* growing cells. Nuclei are represented by hatched area and mesosomes by vesicular structures. It appears that all the mesosomes are connected to the cytoplasmic membrane, that all septa in formation have a mesosome and that all the nuclei are in contact with one or two mesosomes

reconstructions of about twenty cells of *B. subtilis* made by means of serial sections (containing the whole volume of bacteria) showed that all nuclei are in contact with one or two mesosomes (Fig. 18). This contact was found in all the bacteria in a culture and it persists throughout the division cycle. Contact was also maintained during the sporulation process where one of the two mesosomes remains in contact with the sporangial nucleus whereas the other is connected to the spore nucleus and enters the prespore (RYTER and JACOB, 1964).

The mesosomes thus seem to play the role of mediator between the nucleus and the membrane. In general, the area of contact between the mesosome and the nucleus is very large (Fig. 4) and it is not possible to localize the point of attachment with precision. This connection can be identified with greater certainty after the mesosomes have been eliminated from the cytoplasm during protoplast formation (RYTER and JACOB, 1964; RYTER and LANDMAN, 1964).

During its extrusion, the nuclear mesosome seems to pull the nucleus up to the membrane which suggests the existence of a real linkage between DNA and mesosome.

In protoplasts which have no mesosome, the direct connection of nucleus and membrane can also be discerned in a limited number of sections (RYTER and JACOB, 1966; FUHS, 1965). In freshly prepared protoplasts, one can often see a fragment of the mesosomal tube attached to the exterior of the membrane opposite the point where, on its inner face, it is in contact with the nuclear material, as if the insertion point of the mesosomal tube coincides sometimes with the linkage point of the nucleus (RYTER and JACOB, 1966).

Pictures of nuclei in direct contact with the membrane have been obtained under other circumstances, for instance when *B. subtilis* mesosomes have been destroyed by incubation for 2 hours under anaerobic conditions (Fig. 11) (RYTER, 1968). An analogous phenomenon seems to occur during maturation of spores. The mesosome of the prespore, present during the first stages of sporulation, disappears during the formation of spore envelopes (RYTER and JACOB, 1964). The loss of the mesosome is accompanied by the movement of the nucleus from a central position in proximity to the mesosome, to a peripheral position close to the spore membrane (RYTER, 1965). These observations indicate that whenever mesosomes disappear, for whatever reason, the nucleus is brought in direct contact with the membrane. One is led to believe that the point of attachment is located on the mesosomal pocket and that after the disappearance of the mesosomal tubes, the connection is located on the cytoplasmic membrane proper (Fig. 8).

The association between the nucleus and the membrane seems to be relatively well established by morphological studies in *B. subtilis*. It is very possible that it exists also in other Gram-positive bacteria which all possess mesosomes. In Gram negative bacteria, this connection has not been documented in growing bacteria because the mesosomes of these bacteria are generally very poorly developed. A nucleus-membrane contact has however been clearly observed in spheroplasts of *E. coli* (RYTER and JACOB, 1966) and in L-forms of *Salmonella* (RYTER and LANDMAN, unpublished observations).

All of these results suggest that this linkage exists in all bacteria. This view is further supported by the fact that many workers have found an association between DNA and membrane in the course of isolating membrane fractions in both Gram positive and Gram negative bacteria (ANAGNOSTOPOULOS, personal communication; TREMBLAY and NIVELEAU, 1968). It is also confirmed by biochemical and genetic observations (GANESAN and LEDERBERG, 1965; SUEOKA and QUINN, 1968).

The localization of the point of attachment at the mesosome suggests that this organelle also plays a role in nuclear function, or nuclear division. Morphological study of the first division cycle following spore germination has revealed, with the aid of three-dimensional reconstructions, that the connection of the nucleus with the membrane is direct in some cells (RYTER, 1967). Another example of direct attachment has been found in reverting protoplasts.

As has already been pointed out, most of the reverting and reverted protoplasts have no mesosome (RYTER and LANDMAN, 1967; LANDMAN et al., 1968). This absence of mesosomes indicates that the connection between nucleus and membrane must be direct. A number of pictures of reverted protoplasts showing the nucleus in contact with the membrane tend to confrim this assumption (RYTER and LANDMAN, 1967).

If it was true that nuclear mesosomes are indispensable to nuclear function, the cells which are devoid of mesosomes and the protoplasts should not be viable; yet they are able to multiply. Accordingly, it must be conceded that there is no evidence that the nuclear mesosome has any indispensable function for the nucleus.

The process of nuclear division in bacteria and the behaviour of mesosomes during this process is still poorly understood. Observations in the optical

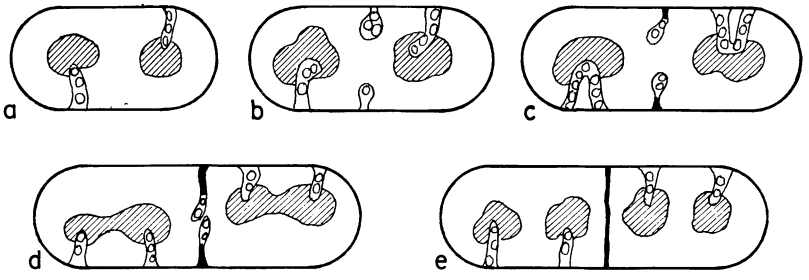

Fig. 19. Scheme of the division cycle deduced from the analysis of three-dimensional reconstructions of about 20 growing cells of *B. subtilis*

microscope made either on stained preparations (ROBINOW, 1956, 1962; LARK et al., 1955) or on living and dividing bacteria (MASON and PAWELSON, 1956) indicate that the nuclear bodies split into two and that the two sister nuclei gradually separate .The analysis of nuclear patterns inthree-dimensionally reconstituted growing cells of *B. subtilis* led to the proposal of a scheme of division which was in accord with these previous observations and provided some new insights into the behaviour of mesosomes (RYTER and JACOB, 1964) (Fig. 19). At the beginning of the division cycle, the two nuclei of the bacterium are each connected to a single mesosome. In the course of DNA replication, as indicated by the increase in nuclear volume, a new mesosome is formed (RYTER, 1967). The two mesosomes then move apart progressively, each carrying with it one of the two sister nuclei.

In bacteria containing only a single nucleus per cell, mesosomes seem to exhibit various kinds of behaviour during the division cycle. In *Streptococcus pyogenes*, the dividing nucleus is in connection with two mesosomes between which the septum of division grows (Fig. 20). In *Mycobacterium phlei* (PETITPREZ et al., 1967) (Fig. 21) each cell has only one mesosome located in the center of the bacterium connected with the nucleus. While the nucleus is dividing, the mesosome participates in septum formation. It finally split in two, the two mesosomes being located one each side of the septum. Then the

organelles move progressively towards the center of the bacteria, carrying the nuclei with them.

ELLAR et al. (1967) have observed in synchronous cultures of *B. megatherium* that the nucleus is in contact with a mesosome located at the pole of the bacterium and with a mesosome involved in transverse septum formation. The association with the mesosome located at the pole of the cell seems to be permanent whereas the attachment to the mesosome in the center of the cell

Fig. 20. *Streptococcus pyogenes* cell in division. The nucleus seems to be attached to two mesosomes between which the septum of division is growing. 116,000×. (Micrograph made by R. COLE, Bethesda)

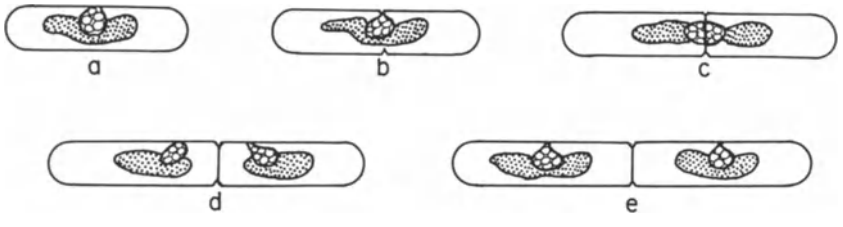

Fig. 21. Division pattern as seen in Mycobaterium phlei

takes place only during septum formation. According to the authors, these observations might suggest that the nuclei are not immediately attached to the membrane after replication.

It has been proposed that the separation of sister nuclei would be achieved by the synthesis of the new membrane between the points of attachment of the two chromosomes (JACOB et al., 1963). The division cycle (Fig. 19), inferred from the analysis of three-dimensionally reconstructed *B. subtilis* and many observations made on other bacteria, seems to agree well with this hypothesis since the two nuclear mesosomes do move apart progressively, each carrying one of the two nuclei. The membrane synthesis presumably occurs between the two mesosomes. Results obtained by FITZ-JAMES (1967, 1968) which

suggest that mesosomes might be the site of membrane synthesis are in agreement with this hypothesis.

Attempts were made to label the membrane in order to study this problem (RYTER, 1967; JACOB et al., 1966) but, although the results of these studies tend to confirm this hypothesis, they did not provide a definite proof and for the time being this question remains opened.

It must, however, be pointed out that the behaviour of the mesosomes and their relation with the nuclei must be interpreted with great caution. The study of mesosome behaviour during the division cyle is complicated by the fact that these organelles can be detected only in the electron microscope, in other words, only in dead bacteria. Their behaviour is deduced from statistical observations of static pictures. The association between nucleus and mesosomes can be established only in serial sections covering the entire volume of the bacteria and, even in these conditions, this association is often difficult to detect with certainty. Moreover, the division cycle cannot be deduced from one or two bacteria reconstructed in three-dimensions; many bacteria must be reconstructed.

For all these reasons, it is obvious that the interpretations of morphological observations made till now can be considered only as working hypotheses.

Conclusions

The respiratory functions which were attributed to mesosomes are confirmed not only by cytochemical studies but also by enzymatic analyses of isolated mesosome reactions. The cytoplasmic membrane also plays an important role in respiration but does not seem to contain exactly the same enzymatic content as the mesosomes. The fact that the mesosomes do not reduce potassium tellurite in the form of needles and also do not disappear under anaerobic conditions (CONTI et al., 1968), in contrast to yeast mitochondria (LINNANE et al., 1962; LUKINS et al., 1966), shows that they are probably rather different from these organelles.

However, these actual data and the knowledge about the respiratory chain are too scanty and fragmentary for any definitive assumption. At the present time it cannot be concluded that mesosomes have a specific role in the respiration of the bacterium, really different from that of the cytoplasmic membrane.

The role of mesosomes in the cell wall synthesis is confirmed by the presence of two enzymes involved in the thecoic acid synthesis. However, since these enzymes are also present in the membrane, mesosomes cannot be considered as organelles indispensable for the wall formation. This is in agreement with the morphologic study of reverting protoplasts which showed that protoplasts are able to manufacture a new cell wall in the absence of mesosomes.

The role of mesosomes in the nuclear function seems to be rather limited. In bacteria which possess several mesosomes per cell, these organelles play the role of intermediary in the linkage between the nucleus and the membrane, but this role does not seem really indispensable since in some cases DNA

replication and nuclear division can take place normally when the nucleus seems directly attached to the membrane.

All these results thus show that mesosomes have many of the functions of the cytoplasmic membrane. For the time being, they cannot be considered as true organelles having a specific function. Probably they represent a means used by bacteria to increase their membrane surface and consequently their enzymatic content. Very recent results obtained by GHOSH and SARGENT (1968) seem to be in agreement with this assumption. These authors observed that in *B. licheniformis*, the formation of mesosomes occurs when the penicillinase synthesis is induced and that this enzyme is bound in large amounts to the mesosomal tubules (SARGENT and GHOSH, 1968). It seems that bacteria form new mesosomes according to their enzymatic needs.

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Recent Problems in the Genetic Code

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With 2 Figures

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Introduction*

The term "genetic code" or "amino acid code" customarily refers to the assignments of the 64 different groups of three consecutive nucleotides in messenger RNA (Table 1). Sixty-one of the 64 are each assigned to an amino acid in the biological synthesis of polypeptide chains and the other three to

* *Abbreviations*: DNA = deoxyribonucleic acid; RNA = ribonucleic acid; mRNA = messenger RNA; tRNA = transfer RNA; A = adenine; C = cytosine; G = guanine; T = thymine; U = uracil; N = A, C, G, T or U; R = A or G; Y = C, T or U; NDP = nucleoside diphosphate; A · T = hydrogen-bonded base pair; I = inosine; Ala-Gly = alanyl-glycine (etc.); Ψ = pseudouridine; ppp- = triphosphate; other abbreviations are in the footnote to Table 8.

Table 1. *The genetic code*

UUU Phenylalanine	CUU Leucine	AUU Isoleucine	GUU Valine
UUC Phenylalanine	CUC Leucine	AUC Isoleucine	GUC Valine
UUA Leucine	CUA Leucine	AUA Isoleucine	GUA Valine
UUG Leucine	CUG Leucine	AUG Methionine	GUG Valine
UCU Serine	CCU Proline	ACU Threonine	GCU Alanine
UCC Serine	CCC Proline	ACC Threonine	GCC Alanine
UCA Serine	CCA Proline	ACA Threonine	GCA Alanine
UCG Serine	CCG Proline	ACG Threonine	GCG Alanine
UAU Tyrosine	CAU Histidine	AAU Asparagine	GAU Aspartic acid
UAC Tyrosine	CAC Histidine	AAC Asparagine	GAC Aspartic acid
UAA Chain Termn.	CAA Glutamine	AAA Lysine	GAA Glutamic acid
UAG Chain Termn.	CAG Glutamine	AAG Lysine	GAG Glutamic acid
UGU Cysteine	CGU Arginine	AGU Serine	GGU Glycine
UGC Cysteine	CGC Arginine	AGC Serine	GGC Glycine
UGA Chain Termn.	CGA Arginine	AGA Arginine	GGA Glycine
UGG Tryptophan	CGG Arginine	AGG Arginine	GGG Glycine

the function of terminating a polypeptide chain by interposing a carboxyl group instead of adding another amino acid. Each such group of three nucleotides is termed a "codon".

Actually, the 61 amino acid codons specify transfer RNA molecules rather than amino acids, but each transfer RNA (tRNA) molecule carries a specific amino acid, and so the code is usually written in terms of the amino acid relationships of 61 of the codons. The codon-tRNA pairing is carried out by hydrogen bonding between codons and a sequence of three nucleotides, an "anti-codon", in the tRNA molecule. This pairing takes place on the surface of a ribosome, and immediately precedes the addition of an amino acid to a growing peptide chain. The synthesis of polypeptides is probably the most crucial of all the chemical processes which are collectively responsible for the existence and perpetuation of life. Problems surrounding the nature and function of the genetic code are therefore of great interest.

In a broader sense, the concept of the genetic code may be expanded to include various relationships between nucleotide sequences and polypeptides. The two groups of compounds, nucleic acids and proteins, have been compared to two languages, each of which must be translatable into the other. The analogy is strengthened by the fact that both are composed in linear sequences of a small number of variables; four in DNA or RNA, and 20 in a protein. There are interfaces, as yet unidentified, at which nucleotide sequences in DNA and RNA recognize and bind to amino acid sequences in enzymes. Some examples of such interfaces are (i) the recognition of a transfer RNA by an aminoacyl tRNA synthetase prior to the charging of the tRNA with an amino acid, (ii) the attachment of RNA polymerase to a C-rich region (Szybalski et al., 1966) of one of the two strands of DNA, preceding the process of transcription, (iii) the modification of various bases at specific locations in molecules of tRNA, ribosomal RNA and DNA, (iv) the formation of ribosomes by

combination of proteins with RNA, (v) the initiation of replication of DNA by its polymerase.

In each of these examples, a protein selects and binds to a sequence of nucleotides, but the process is so obscure that not even the length of either of the two interacting regions is known.

Establishment of Codon Assignments

The establishment of the assignments in the genetic code is buttressed by several converging lines of evidence. These include:

(i) Production of polypeptides in cell-free systems primed with synthetic polyribonucleotides of random sequence.

(ii) Identification of single amino acid mutations occurring spontaneously in proteins.

(iii) Production of single amino acid mutations and their revertants by chemical mutagenesis.

(iv) Sequential analysis of the anticodon loops of tRNA molecules.

(v) Wobble pairing between anticodons in tRNA and synthetic codons.

(vi) Production of polypeptides in cell-free systems using polyribonucleotides with defined repeating sequences, and sequential analysis of the resultant polypeptides.

(vii) Identification of polypeptide changes caused by frameshift mutations.

(viii) Correlation between the composition of proteins and the composition of DNA.

(ix) The pattern of amino acid replacements in homologous proteins during evolution.

(x) Suppressor mutations in anticodons.

Synthetic Polyribonucleotides of Random Sequence

In the initial investigations of the code, the assignments of messenger RNA triplets (codons) to amino acids were made by means of experiments with *E. coli*. At first, synthetic polyribonucleotides of known composition, but with unknown and random sequences, prepared by the action of polynucleotide phosphorylase, were used. This enzyme under certain conditions has a synthetic action when supplied with ribonucleoside diphosphates as substrates, according to the following reaction:

$$\mathrm{nNDP} \rightarrow (\mathrm{NMP})_n + \mathrm{nP}_i$$

Under other conditions, the reaction proceeds in the opposite direction, i.e., hydrolysis of RNA to diphosphates by phosphorolysis, and it is this latter reaction that is thought to predominate for the enzyme *in vivo*. The deciphering of the code was facilitated by the finding of an underlying pattern of specificity for two bases of a codon, even before the sequences of different bases in codons were known. It became evident, for example, that threonine codons contained A and C, that some serine codons contained U and C and

others A and G, and that phenylalanine could be coded by 3U or by 2U, 1C. The findings that resulted from this experimental approach were summarized by NIRENBERG et al. (1963), by SPEYER et al. (1963), and were reviewed by JUKES (1966).

These studies were followed by binding experiments in which trinucleotides, attached to ribosomes, were found to attract and bind specific tRNA molecules and by experiments in which synthetic polyribonucleotides of known sequence were used as messenger RNA to synthesize polypeptides in cell-free amino-acid-incorporating systems, usually prepared from *E. coli*.

Single Amino Acid Mutations

INGRAM (1957) identified the mutation in the variant β chain in hemoglobin S as a change of glutamic acid to valine at residue 6. This is a *point mutation* and is brought about by a change of an A · T pair to T · A pair in DNA. The ensuing years have seen identification of many such "single-amino acid" changes in mutant proteins. These are summarized in Table 2. Of 75 possible single-amino-acid changes corresponding to single-base changes in codons, 54 have been identified. Only one occurrence of one change corresponding to a two-base codon alteration has been reported; methionine to glutamic acid in tryptophan synthetase A protein. Most of the mutations in the coat protein of tobacco mosaic virus listed were produced by the action of chemical mutagens on the virus. Only 21 of the possible single amino acid mutations caused by single-base changes in codons are still undiscovered after a mere eleven years of research in this comparatively laborious field. The results in Table 2 substantiate the codon assignments in Table 1 and support the concept of universality of the genetic code.

Wobble Pairing and Anticodon Loops in tRNA

The pattern of the amino acid code is such that it gave rise to the early "doublet code" proposal by ROBERTS (1962), in which two bases specify an amino acid, and to the "two-and-a-half letter code" concept advanced by ECK (1963). It was not until the base sequence of a tRNA molecule had been completely mapped by HOLLEY et al. (1965) that the underlying reason for the distribution of 64 three-base codons among 21 functions was brought to light.

The presence of many "unusual", or modified, bases in tRNA is one of the most striking phenomena in nucleic acid chemistry. Scattered throughout the sequence of nucleotides in tRNA molecules are various bases that carry extra methyl, O-methyl, thio, isopentenyl and other groups which are present as the result of the action of specific enzymes. The number and complexity of the modified bases continues to grow as further analyses of tRNA molecules are made. Very little is known of the functions of the modified bases. Their disposition and types vary from molecule to molecule of tRNA, and from species to species, dependent upon tRNA-specific and species-specific enzymes. The

Table 2. *Single amino acid mutations in proteins*

Mutational interchange between	and	Codons corresponding	Proteins
Alanine	Aspartic acid	GCY; GAY	Hb, TMV, TS
	Glutamic acid	GCR; GAR	Hb, TS
	Glycine	GCN; GGN	TMV
	Threonine	GCN; ACN	TMV
	Valine	GCN; GUN	AP, LG, TMV, TS
Arginine	Glutamine	CGR; CAR	Hb, TMV
	Glycine	CGN; GGN or AGR; GGR	Hb, TMV, TS
	Histidine	CGY, CAY	Hb
	Isoleucine	AGA; AUA	TS
	Leucine	CGN; CUN	TS
	Lysine	AGR; AAR	TMV
	Proline	CGN ; CCN	Hb
	Serine	AGR; AGY	TS
	Threonine	AGN; ACN	TS
	Tryptophan	AGG; UGG or CGG; UGG	MD
Asparagine	Aspartic acid	AAY; GAY	Hb, GDH
	Lysine	AAY; AAR	Hb, TMV
	Serine	AAY; AGY	TMV
	Threonine	AAY; ACY	Hb
	Tyrosine	AAY; UAY	Hb
Aspartic acid	Glycine	GAY; GGY	Hb, LG, TMV, TF, TS
	Histidine	GAY; CAY	Hb
	Valine	GAY; GUY	TMV
Cysteine	Glycine	UGY; GGY	TS
	Tyrosine	UGY; UAY	TS
	Tryptophan	UGY; UGG	YMP
	Serine	UGY; UCY	Hb
Glutamic acid	Aspartic acid	GAR; GAY	TMV, TS
	Glycine	GAR; GGR	Hb, TMV, TS
	Lysine	GAR; AAR	Hb
	Valine	GAR; GUR	Hb
	Methionine[a]	GAG; AUG	TS
Glutamine	Glutamic acid	CAR; GAR	Hb
	Histidine	CAR; CAY	LG, Hb
	Lysine	CAR; AAR	Hb
Glycine	Serine	GGY; AGY	TMV
	Valine	GGR; GUR	TS
Histidine	Tyrosine	CAY; UAY	Hb
Isoleucine	Methionine	AUA; AUG	TMV
	Serine	AUY; AGY	TS
	Threonine	AUY; ACY or AUA; ACA	TMV, TS
	Valine	AUY; GUY or AUA; GUA	TMV

Table 2. (Continued)

Mutational interchange between	and	Codons corresponding	Proteins
Leucine	Methionine	YUG; AUG	CC
	Phenylalanine	CUY; UUY or UUR; UUY	TMV
	Proline	CUN; CCN	Hb, TMV
	Serine	UUR; UCR	TMV, TS
Lysine	Threonine	AAR; ACR	Hb
Methionine	Threonine	AUG; ACG	TMV
	Valine	AUG; GUG	TMV, Hb
Phenylalanine	Serine	UUY; UCY	Hb, TMV
	Tyrosine	UUY; UAY	TMV, Hb
	Valine	UUY; GUY	Hb
Proline	Serine	CCN; UCN	TMV
	Threonine	CCN; ACN	TMV

Abbreviations. CC = Cytochrome *c*; Hb = hemoglobin; LG = lactoglobulin; MC = malic dehydrogenase; TF = transferrin; TMV = Tobacco mosaic virus coat protein; TS = *E. coli* tryptophan synthetase A protein; YMP = yeast mitochondrial protein; N = A, C, G or U; R = A or G; Y = C or U.

[a] 2-base change.

methylation of tRNA is also dependent upon a supply of methionine (MANDEL and BOREK, 1963).

One of the modified bases, inosine, which was discovered in tRNA by HALL (1963), has a coding function when it occurs in the third position of the anticodon loop. This was perceived by CRICK (1966). He drew attention to the presence of IGC in alanine tRNA, IGA in serine tRNA, and IAC in valine tRNA. These three amino acids all have codes in which the third position of codons can be occupied by any of the four bases. Stereochemical considerations led Crick to predict that cytidine, uridine or adenosine (C, U or A) in mRNA could pair with inosine in the first position of the anticodon, assuming that there was a certain amount of extra space, or "wobble", between the paired bases. Pairings between G in the anticodon and U or C in the codon; and U in the anticodon and A or G in the codon were also predicted for the first nucleotide in the anticodon. So far, inosine has been found present in the first anticodon position of six tRNAs, all of which are for amino acids with codons terminating in C, U or A. Inosine has not been found to occur in positions 2 or 3 in anticodons, although methylinosine is present in the position next to the anticodon in yeast alanine tRNA. The function of inosine in anticodons seems to have been established by the wobble hypothesis.

Since RNA molecules are assumed to be synthesized from four unmodified bases in the form of the nucleoside triphosphates of A, C, G and U, it follows that inosine in anticodons is produced by enzymatic deamination of adenosine in tRNA molecules. Evidence for this was obtained by KAMMEN (1969) who

Table 3. *Nucleotide binding by tRNAs*

tRNA	Trinucleotides bound	Indicated anticodon	Reference
EC serine I	UCU; UCC	GGA	1
EC serine II	UCA; UCG	UGA	1
EC serine III	AGU, AGC	GCU	1
EC, GP serine I	AGU; AGC	GCU	5
EC serine II	UCU; UCC	GGA	5
EC serine III	UCU; UCA; UCG	?	5
GP serine II	UCU; UCC; UCA	IGA	5
GP serine III	UCG	CGA	5
EC serine suppressor	UAG	CUA	5
Y, EC, GP arginine I	CGA; CGU; CGC	ICG	1, 5
EC, GP arginine II	CGG	CCG	5
GP arginine III	CGA; CGG	UCG	5
Y arginine II	AGA; AGG	UCU	1
GP arginine IV	AGG	CCU	5
EC isoleucine I	not by AUU or AUC	?	1
EC isoleucine II	AUU; AUC	CAU; IAU	1
EC isoleucine III	AUU; AUC	GAU; IAU	1
EC isoleucine	AUU; AUC; not AUA	GAU	5
GP isoleucine I	AUU; AUC; AUA	IAU	5
TY isoleucine	not reported	IAU found	6
Y phenylalanine	UUU; UUC	GAU, IAU	1
EC alanine I	GCA; GCG	UGC	1
Y alanine I	GCU; GCC; GCA	IGC	1
Y alanine II	GCA; GCG	UGC	7
EC tryptophan	UGG	CCA	1
EC methionine	AUG	CAU	1
EC valine I	GUA; GUG	UAC	1
EC valine II	GUU; GUU	GAC	1, 2
Y valine I	GUU; GUA; GUC	IAC	1
Y glycine I	GGA; GGG	UCC	1
Y glycine II	GGU; GGC	GCC	1
EC leucine I	CUG	CAG	3
EC leucine III	UUG	CAA	1
EC cysteine I	UGU; UGC	GCA	5
EC cysteine II	UGU; UGC; UGA	?	5
GP cysteine I	UGU; UGC (UGA)	?	5
GP cysteine II	UGU; UGC (UGA)	?	5
EC, GP lysine I	AAA; AAG	UUU	5
GP lysine II	AAG	CUU	5
EC, GP methionine II	AUG	CAU	5
EC formylmethionine	AUG; GUG; UUG	CAU	4
EC formylmethionine	AUG; GUG (UUG, CUG)	CAU	5
GP formylmethionine	AUG; GUG	CAU	5
EC tyrosine suppressor	UAG	CUA	
GP threonine	ACU; ACC; ACA	ICU	5

Abbreviations. EC = *E. coli*; GP = guinea pig; Y = yeast; TY = *Torulopsis* yeast.
1 = SÖLL et al., 1966; 2 = KELLOGG et al., 1966; 3 = SÖLL et al., 1965; 4 = CLARK and MARCKER, 1966; 5 = CASKEY et al., 1968; 6 = TAKEMURA et al., 1969; 7 = CHAMBERS et al., 1969; 8 = GOODMAN et al., 1968.

Table 3a. *Codon-Anticodon pairing. Codons and Possible Anticodons in terms of the "wobble" theory* (CRICK, 1966). *Codon-Anticodon pairing is indicated by arrows. Identified anticodons are italicized.* **G* = *2'-OMeG*; †*G* = *modified G*

Amino acids	Codons	Anticodons	Amino acids	Codons	Anti-codons
Phenylalanine	UUU		Isoleucine	AUU	*IAU*
Phenylalanine	UUC	**GAA*	Isoleucine	AUC	GAU
Leucine	UUA	UAA	Isoleucine	AUA	
Leucine	UUG	CAA	Methionine	AUG	*CAU*
Serine	UCU	*IGA*	Threonine	ACU	IGU
Serine	UCC	GGA	Threonine	ACC	GGU
Serine	UCA	UGA	Threonine	ACA	UGU
Serine	UCG	CGA	Threonine	ACG	CGU
Tyrosine	UAU		Asparagine	AAU	
Tyrosine	UAC	*GΨA*, †*GUA*	Asparagine	AAC	GUU
Chain Term.	UAA	UUA	Lysine	AAA	UUU
Chain Term.	UAG	*CUA*	Lysine	AAG	CUU
Cysteine	UGU		Serine	AGU	
Cysteine	UGC	GCA	Serine	AGC	GCU
Chain Term.	UGA		Arginine	AGA	UCU
Tryptophan	UGG	CCA	Arginine	AGG	CCU
Leucine	CUU	IAG	Valine	GUU	*IAC*
Leucine	CUC	GAG	Valine	GUC	GAC
Leucine	CUA	UAG	Valine	GUA	UAC
Leucine	CUG	CAG	Valine	GUG	CAC
Proline	CCU	IGG	Alanine	GCU	*IGC*
Proline	CCC	GGG	Alanine	GCC	GGC
Proline	CCA	UGG	Alanine	GCA	UGC
Proline	CCG	CCC	Alanine	GCG	CGC
Histidine	CAU		Aspartic acid	GAU	
Histidine	CAC	GUG	Aspartic acid	GAC	GUC
Glutamine	CAA	UUG	Glutamic acid	GAA	UUC
Glutamine	CAG	CUG	Glutamic acid	GAG	CUC
Arginine	CGU	ICG	Glycine	GGU	ICC
Arginine	CGC	GCG	Glycine	GGC	GCC
Arginine	CGA	UCG	Glycine	GGA	UCC
Arginine	CGG	CCG	Glycine	GGG	CCC

found that adenosine, but not inosine, was incorporated into *E. coli* tRNA as shown by isotope dilution.

The other tRNA base that may be involved in coding functions is the adenosine adjoining the anticodon. This adenosine is in a modified form in 12 of the 14 identified tRNAs. DUBE et al. (1968) suggested that the unmodified A in *E. coli*, N-formyl methionine tRNA (Table 8), enables wobble to take place between the third position of its anticodon, CAU, and the first position in the codon, so that this tRNA binds both AUG and GUG codons, while other tRNAs are prevented from ambiguous base pairing in the third anticodon position by the presence of side chains on the adjoining adenine residue. However, yeast formyl methionine tRNA has a modified adenosine adjoining the anticodon. The identified bases in this position include methyl-

Table 4. *Incorporation of amino acids into polypeptides as directed by ribopolynucleotides with repeating sequences*

Repeating sequence	Codons in sequence	Amino acids incorporated into polypeptides[a]
UC	UCU; CUC	Ser-Leu
UG	UGU; GUG	Cys-Val[b]
AG	AGA; GAG	Arg-Glu
AC	ACA; CAC	Thr-His
UAC	UAC; ACU; CUA	Tyr, Thr, Leu
UUC	UUC; UCU; CUU	Phe, Ser, Leu
UUG	UUG; UGU; GUU	Leu, Cys, Val
AUC	AUC; UCA; CAU	Ile, Ser, His
AAG	AAG; AGA; GAA	Lys, Arg, Glu
CAA	CAA; ACA; AAC	Gln, Thr, Asn
GUA	GUA; UAG; AGU	Val, Ser[c]
GAU	GAU; AUG; UGA	Asp, Met[c]
UAUC	UAU; CUA; UCU; AUC	Tyr-Leu-Ser-Ile
UUAC	UUA; CUU; ACU; UAC	Leu-Leu-Thr-Tyr
GUAA, GAUA	All sequences contain chain terminating codons	None, None

[a] Alternating polypeptides were produced by repeating sequences of 2 and 4 ribonucleotides; homopolypeptides by repeating sequences of 3 nucleotides.

[b] N-formyl Met-(Val-Cys)$_n$ was produced when N-formylmethionine tRNA was supplied in addition to valine and cysteine tRNAs.

[c] UAG and UGA do not code for amino acids (Table 1).

Source. KHORANA et al. (1966); KÖSSEL et al. (1967).

inosine, isopentenyl adenosine and methyl-thio isopentenyl adenosine. Isopentenyl adenosine is a plant hormone or "cytokinin" (HALL et al., 1967).

N-formyl methionine tRNA apparently also pairs weakly with the codon GUA, but the interpretation of this in terms of the wobble pattern is not clear.

Does wobble pairing occur in the same pattern in all species? This point is being debated. Most investigators have approached the question by examining the trinucleotide binding pattern of purified tRNAs. The results are summarized in Table 3. The importance of using homogeneous tRNAs for such studies is obvious, and has been stressed by SÖLL et al. (1966). In nearly all cases, the binding of tRNAs by synthetic trinucleotides is in agreement with wobble pairing (Table 3a). The major exceptions are three cysteine tRNAs which have been reported to pair with UGA (which is not a codon for cysteine) as well as with UGU and UGC.

In terms of the wobble theory, tRNAs with the anticodons UCA and UAU cannot exist; UCA would pair with both UGG (tryptophan) and UGA (chain termination) while UAU would pair with both AUG (methionine) and AUA (isoleucine). Such ambiguity would probably be lethal.

KHORANA and his collaborators (1966) in a monumental series of experiments, have prepared synthetic polydeoxyribonucleotides and polyribo-

nucleotides of known sequence and have used these compounds as messengers for the synthesis of polypeptides, by cell-free systems obtained from *E. coli*. The results are in Table 4. The composition and sequence of 28 codons and the direction of reading of the genetic message are shown by these results, which leave little doubt as to the general nature of the assignments of mRNA triplets in the genetic code. For a description of the experimental procedures, the reader is referred to the articles by KHORANA et al. (1966) and KÖSSEL et al. (1967).

These results with synthetic polyribonucleotides are in excellent agreement with *in vivo* findings made with acridine-induced mutations of bacteriophage T4 lysozyme (TERZAGHI et al., 1966). Two lysozyme-negative acridine mutants were recombined to produce a new mutant, which was lysozyme-positive, with about 50 per cent of the lysozyme activity of wild type. The lysozymes were isolated from the wild type and the new mutant and were digested with trypsin and chymotrypsin. Two peptides were found to differ between the wild type and mutant. The differences in the respective sequences were established by overlapping peptides, as follows:

Wild:	
Chymotryptic	Thr–Lys–Ser–Pro–Ser–Leu–Asn
Tryptic	Ser–Pro–Ser–Leu–Asn–Ala–Ala–Lys
New Mutant	
Chymotryptic	Thr–Lys–Val–His–His–Leu
Tryptic	Val–His–His–Leu–Met–Ala–Ala–Lys

As a result of the genetic recombinations, the sequence Ser–Pro–Ser–Leu–Asn changed to Val–His–His–Leu–Met. This corresponds to the deletion of an adenine at the left of the section coding for the sequence and the insertion of a guanine at the right, with only one solution in terms of the code as follows:

```
...A-A-R-A-G-U-C-C-A-U-C-A-C-U-U-A-A-U-G-C-N...
    Lys     Ser     Pro     Ser     Leu     Asn     Ala
...A-A-R-G-U-C-C-A-U-C-A-C-U-U-A-A-U-G-G-C-N...
    Lys     Val     His     His     Leu     Met     Ala
```

The results also demonstrate that the genetic message is translated from left to right in protein synthesis, in terms of the convention used in writing the abbreviated formula for RNA, as 3′- to 5′-phosphate diester linkages in the examples shown above. The tryptic peptide ends in ...Ala–Ala–Lys, terminating with the carboxyl group of lysine, so it is evident that this was the last amino acid to be incorporated in this peptide, and its synthesis took place by reading the mRNA from left to right.

Composition of Proteins and DNA

As shown by SUEOKA (1961), there is a tendency for the composition of bacterial proteins to be correlated to a limited extent with the G + C content of bacterial DNA. There is a wide variation in the G + C content of bacterial DNA. Some species have as little as 34 % G + C in their DNA, and others as

much as 72%. Intermediate values were found throughout this range when various microorganisms were examined (MARMUR et al., 1963). A precise interpretation of Sueoka's data is not possible because, in the analyses, aspartic and glutamic acids were not distinguished from the corresponding amides. However, the results showed definitely that alanine, glycine, proline and arginine increased as G + C increased, and that isoleucine, lysine, phenylalanine, tyrosine and methionine increased as G + C lessened. The differences in amino acids were much smaller, percentage-wise, than the shifts in base composition. It was suggested (JUKES, 1966) that this disparity between amino-acid differences and differences in G + C was explainable in terms of the third positions of codons being occupied by A + U in the 34% G + C species, and by G + C in the 72% G + C species. Experimental evidence for this was provided by COX and YANOFSKY (1967). They found that the Treffers' mutator gene, which favors the change A · T→C · G in DNA, apparently "filled up" the ambiguous third positions of codons with C and G, and then exerted mutational pressure that tended to displace amino acids with codons high in G and C. Examples would be the replacement of isoleucine by valine, or threonine by alanine.

The distribution of amino acid replacements in homologous proteins during evolution can be studied by comparing them in terms of the amino acid code. It is assumed that such replacements originate as point mutations which are the result of randomly-distributed base replacements in DNA (JUKES, 1963). With this assumption, it follows that in two homologous proteins that differ only in a few amino-acid residues, most of the differences will represent single-base changes. As the number of differences increases, the base replacements will be more numerous, so that more and more cistrons will be "hit twice" and there will be a steady increase in the number of amino acid replacements corresponding to two-base changes. These predictions are realized, and are exemplified in Table 5. Myoglobin and the hemoglobin chains became separated by gene duplication hundreds of millions of years ago. The α chain separated from the β and γ chains later, followed by the separation of the β and γ chains and then by the β and δ separation (INGRAM, 1963). The various vertebrates listed in Table 5 are in descending order of evolutionary separation, starting with the lamprey.

One suppressor mutation has been identified in an anticodon. This is GUA to CUA in tyrosine tRNA. Tyrosine suppressor tRNA pairs with the UAG anticodon, which signals chain termination (GOODMAN et al., 1968).

Special Problems in Codon Assignments

a) *AUA*. The assignment of AUA to isoleucine instead of to methionine is an exception to the usual pattern of the other codon assignments (Table 1). The failure of *E. coli* isoleucine-tRNA to bind to the trinucleotide AUA tended to engender hesitation on the part of various authors to accepting this assignment. Despite this lack of affinity between AUA and isoleucine tRNA, *E. coli* systems were shown to incorporate isoleucine in synthetic

Table 5. *Minimum base changes per codon in the relationship between human α, β, γ and σ hemoglobin chains and myoglobin, between human α and lamprey hemoglobin chains, between human and horse α and β hemoglobin chains, human and carp α hemoglobin chains, and human and rhesus monkey β chains*

Comparison	Minimum differences per codon					Ratio of single-base changes to 2 or 3 base changes
	sites compared	none	1	2	3	
Myoglobin: α human	139	38	56	44	1	1.2
Myoglobin: β human	143	37	56	48	2	1.1
Myoglobin: γ human	143	37	59	46	1	1.3
α human: lamprey	100[a]	38	40	22	0	1.8
α human: β human	139	64	53	22	0	2.4
α human: γ human	139	59	55	25	0	2.2
α human: α carp	140	73	42	25	0	1.7
β human: γ human	146	107	29	10	0	2.9
β human: β horse	146	121	18	7	0	2.6
α human: α horse	141	124	12	5	0	2.4
β human: δ human	146	136	9	1	0	9.0
β human: β rhesus monkey	146	138	6	2	0	3.0

[a] Only the first 100 residues of lamprey hemoglobin have been sequenced.

polyribonucleotides terminating with AUA (STANLEY et al., 1966), and mammalian tRNA binds well with AUA. In addition, the series of tryptophan synthetase A protein mutants explored by YANOFSKY included an arginine-isoleucine interchange, which can be explained as a single-base change only by AGA→AUA. Finally, the identification of IAU as an isoleucine anticodon in *Torulopsis utilis* (TAKEMURA et al., 1969) appears to remove the last vestige of doubt from the assignment of AUA to isoleucine.

b) *UGA*. This was the last of the codons to be assigned. Opinions as to its role at first wavered between cysteine and tryptophan. There was evidence that the incorporation of tryptophan by U, I, A polyribonucleotides of random sequence was at too low a rate to account for a second tryptophan codon in addition to UGG (TAKANAMI and YAN, 1965). A slight increase above background in the binding of guinea-pig cysteine tRNA by UGA was reported by SÖLL et al. (1965) and a more marked effect was noted by MARSHALL et al. (1967). In contrast, the repeating copolymer $(GAU)_n$ failed to produce incorporation of cysteine or tryptophan into polypeptides in an *E. coli* system (MORGAN et al., 1966), although polyaspartic acid and polymethionine were found. These findings, together with evidence from mutants (BRENNER et al., 1967; WEIGERT et al., 1967), have established UGA as a chain-terminating codon, although its occurrence is considered to be much rarer than that of UAA. No UCA anticodon is known.

c) *UAG and UAA*. These were well-established as chain-terminating codons in studies reported by WEIGERT and GAREN (1965) and BRENNER et al.

(1965). The names "amber" and "ochre" for these two codons persist in the literature. "Amber" is derived from BERNSTEIN, a graduate student who collaborated in the preparation of mutants, and "ochre" was coined by BRENNER without explanation. The terms apparently are intended to convey a cabalistic aura. Regardless of terminology, these two chain-terminating codons are not known to be paired by the corresponding complementary anticodons CUA and UUA, although suppressor tRNAs are known to exist with the anticodon CUA and presumably with UUA.

d) *The CpG problem.* JOSSE et al. (1961) devised a classical procedure for measuring the number of adjoining pairs of bases in DNA molecules, termed the "nearest-neighbor" method. The procedure is as follows: Synthesis of DNA is carried out with DNA polymerase, using a sample of native DNA as a template and a mixture of the four standard deoxynucleoside triphosphates (of A, C, G and T) as substrate. One of the four has ^{32}P esterified to deoxyribose in the 5′-position, e.g. ppp*T, so that DNA is produced that contains Ap*T, Cp*T, Gp*T and Tp*T. The products are then hydrolyzed with micrococcal deoxyribonuclease and spleen phosphodiesterase. This breaks the 5′-linkage, so that Ap*, Cp*, Gp* and Tp* are produced. These are determined quantitatively, and the amounts of ApT, CpT, GpT and TpT in the synthesized DNA are thus revealed. The proportions are identical with those present in the original template. The steps are repeated with ppp*C, ppp*G and ppp*A and the proportions of the other twelve possible doublets are calculated from the results. JOSSE et al. (1961) and SWARTZ et al. (1962) examined a number of DNAs by this procedure, and compared the frequencies of the various doublets with the values predicted from random association of the bases present in each DNA that was tested. The doublet TpA was present in amounts below the predicted levels, as would be expected from the fact that it is transcribed into the two chain-terminating codons UAA and UAG, which are necessarily rarer than codons for amino acids. GpC was more frequent than would be anticipated from the random level in bacterial DNA, which is in accordance with the high levels of alanine, 10% to 16%, in bacterial proteins (SUEOKA, 1961). The most conspicuous disparity was CpG in animal DNA, especially mammalian DNA. CpG was present at only about one-third or less of the frequency predicted from randomness, which would lead to equal amounts of CpG and GpC. The percentages in human DNA were CpG, 1.0; GpC, 4.3; rabbit, 1.3 and 4.8; chicken, 1.1 and 5.2; bovine, 1.5 and 4.5; salmon, 1.7 and 4.0; starfish, 2.5 and 3.8; sea urchin, 2.1 and 3.3; *Tetrahymena*, 0.7 and 2.0. The GpC percentages, in contrast to the amounts of CpG, were close to the random values.

In terms of messenger RNA codons, this paucity of CpG should be reflected, of course, in a scarcity of the four codons CGN (arginine) and the single codons UCG (serine) ACG (threonine) GCG (alanine) CCG (proline); also of sixteen pairs of adjoining codons of the general type NNCGNN. Any scarcity of the single NCG codons or of adjoining codon pairs NNCGNN would be difficult or impossible to detect from protein analyses because of alternate

codons for the codons involved. The case of arginine is different, however, because four of its six codons contain the sequence CG.

The frequence of occurrence of the various amino acids in a large number of vertebrate (mostly mammalian) proteins and polypeptides was summarized and tabulated by KING and JUKES (1969). The distribution of the 5,492 amino

Table 6. *Amino acid frequencies among 5,492 residues in 53 vertebrate polypeptides, compared with random permutations of nucleic acid bases*

Amino acid	Codons	Number of occurrences	Observed frequencies per cent	Frequencies from code
Serine	UCU; UCA UCC; UCG AGU; AGC	443	8.1	8.6
Leucine	CUU; CUA CUC; CUG UUA; UUG	417	7.6	7.9
Glycine	GGU; GGA GGC; GGG	408	7.4	7.2
Alanine	GCU; GCA GCC; GCG	406	7.4	6.0
Lysine	AAA; AAG	394	7.2	5.5
Valine	GUU; GUA GUC; GUG	375	6.8	6.1
Threonine	ACU; ACA ACC; ACG	339	6.2	6.9
Aspartic acid	GAU; GAC	322	5.9	3.6
Glutamic acid	GAA; GAG	317	5.8	4.7
Proline	CCU; CCA CCC; CCG	275	5.0	5.0
Asparagine	AAU; AAC	243	4.4	4.2
Arginine	CGU; CGA CGC; CGG AGA; AGG	229	4.2	10.7
Phenylalanine	UUU; UUC	222	4.0	2.2
Isoleucine	AUU; AUA AUC	209	3.8	5.2
Glutamine	CAA; CAG	203	3.7	3.9
Tyrosine	UAU; UAC	183	3.3	3.1
Cysteine	UGU; UGC	181	3.3	2.6
Histidine	CAU; CAC	158	2.9	3.0
Methionine	AUG	96	1.8	1.8
Tryptophan	UGG	72	1.3	1.6

acid residues in the series is shown in Table 6. With the notable exception of arginine, the commonest amino acids are those that have six codons apiece. Four of the next five most common have four codons each. The least frequent are methionine and tryptophan, each of which has only one codon. The exceptional case of arginine presumably results from the fact that CpG is much rarer than would be anticipated from the C + G content of animal DNA (SUBAK-SHARPE et al., 1966a).

From the observed frequencies of amino acids as set forth in Table 5, KING and JUKES calculated the expected frequencies as follows: For the first two positions of the codons making up the corresponding messenger RNA, the base composition is: uracil, 22.0%; adenine, 30.3%; cytosine, 21.7% and guanine, 26.1%.

It was assumed that the distribution of third-position bases was the same as that of the first two positions. The hypothesis was then tested: are the amino-acid residues distributed according to random permutations of the nucleic acid bases?

For example, the codons for tyrosine are UAU and UAC. From the messenger RNA base composition, the random expectation for the frequency of tyrosine is (0.220) (0.303) (0.220) + (0.220) (0.303) (0.217) = 0.0292. Since not all codons specify amino acids, this should be multiplied by a correction factor of 1.057. The expected frequency of tyrosine is thus 3.09%; the observed frequency is 3.33%. Arginine is present at only 39% of the expected frequency, which may be compared with the finding that CpG occurs at 25% of the expected frequency in human DNA.

What brings about the scarcity of CpG in animal DNA? It does not seem probable that DNA polymerase shows an enzymatic distaste for synthesizing the CpG linkage, because the CpG doublet is present in all animal DNAs that have been examined, even though the amounts are lower than anticipated from the base composition. Furthermore, there is evidence from mutational data that CGN arginine codons are used in the synthesis of human hemoglobin, because, as discussed below, mutations have been discovered which require such codons for single-base changes. We suggest that the reason for the scarcity of CpG is because the amount of arginine that can be tolerated in animal proteins is less than the amount which would result from the occurrence of all six arginine codons in the frequency resulting from the randomization of the bases which compose these codons. In simple terms, there are just too many codons in the genetic code table for arginine and, as a result, many mutations to arginine fail to persist, so that the content of CpG in DNA has been lowered by natural selection. This explanation should lead to the prediction that the arginine codons AGA and AGG should also occur in animal DNA at subnormal amounts. For two reasons, however, it is difficult to decide this point; first, CpT is complementary to ApG so that one cannot decide which of the two doublets is transcribed (in contrast, CpG is always transcribed as CpG); second, two of the four codons starting with ApG are serine codons.

It was proposed by SUBAK-SHARPE et al. (1966a) that the scarcity of CpG could lead to difficulties for the existence of some large mammalian viruses with a high content of G + C and with a nearest neighbor distribution which differed markedly from that of the host.

In contrast, the DNA of certain small viruses, including polyoma and Shope papilloma viruses, had nearest neighbor patterns which corresponded closely to those of their hosts, a finding which suggests that these viruses originated by "escaping" from the host as a fragment of its genome.

Examples of the large viruses are herpes and pseudorabies, which have CpG contents of approximately 11 % and 15 %, respectively, as compared with about 1 % in human DNA. SUBAK-SHARPE proposed this disparity would make it necessary for the viral DNA to contain cistrons for transfer RNAs which were not supplied by the host, or were supplied only in small amounts.

SUBAK-SHARPE et al. (1966b) extracted tRNA from hamster kidney cells in tissue culture after infection with herpes virus, and compared the tRNA preparation with the corresponding fraction obtained from non-infected cells. The elution patterns of arginine tRNAs from the two preparations showed marked differences, and differences were also found when T1 ribonuclease hydrolysates of the two arginine tRNAs were subjected to chromatographic fractionation of the resultant oligonucleotides. The authors concluded that at least one, and probably two, new arginine tRNAs appeared following infection with herpes virus.

These findings draw attention to the relation of the quantitative supply of tRNAs as a limiting factor in the rate of protein synthesis. It seems certain that mammalian cells contain arginine tRNAs complementary with CGN codons. CASKEY et al. (1968) have shown that arginine tRNAs pairing with such codons are present in the guinea pig. Mutations of histidine to arginine ($\beta 63$); arginine to glutamine and leucine ($\alpha 92$) proline to arginine ($\alpha 114$ and $\beta 58$) leucine to arginine ($\alpha 14$) glutamine to arginine ($\alpha 54$) have been discovered in human hemoglobin, all of which correspond to single base changes to or from CGN codons as follows: CAY→CGY; CGR→CAR and CUR; CCN→CGN; CUN→CGN; and CAR→CGR. These changes require two-base or three-base changes if AGR is involved, which may be ruled out, since all 91 single-amino-acid mutations in hemoglobin, listed in the summary by PERUTZ and LEHMANN (1968), correspond to single-base codon changes.

One other possibility must be considered with respect to the small or large proportion of any doublet in the total DNA as compared with the proportion anticipated from the codons that represent the proteins of the organism: very little of the DNA of higher organisms is transcribed into messenger RNA.

Chain Termination

It was found by CASKEY et al. (1968) that formyl methionine was released from its tRNA, bound to ribosomes, when any of the trinucleotides UAA, UAG or UGA were added. The procedure that demonstrated this effect consisted of *E. coli* 70S ribosomes, the trinucleotide AUG, and f-Met tRNA

labeled with tritium in the formyl group. The presence of AUG causes binding of f-Met tRNA to ribosomes. Addition of any of the chain-terminating codons, in the form of a trinucleotide, together with a preparation of the "release factor" described by CAPECCHI (1967), set formylmethionine free from its attachment to the tRNA-ribosome complex, apparently by breaking the ester linkage between methionine and tRNA.

The only trinucleotides found to have this effect were UAA, UAG and UGA. This encouraged the authors to regard the procedure as a test for the chain-termination mechanism, and especially as a means of studying the "release factor" (*R*). *R* was inactivated by trypsin, N-ethylmaleimide or by incubation at 100°, but not by T1 ribonuclease, diisopropylfluorophosphate, or pancreatic ribonuclease. The possibility that *R* contains a nucleotide group is not excluded, however, the findings suggest that a protein (rather than a special tRNA) recognizes and binds to a chain-terminating codon, and attacks the bond between the last amino acid in the polypeptide chain and the tRNA which has paired with the codon immediately preceding the chain-terminating codon. SCOLNICK et al., 1968, have since demonstrated that *R* consisted of two components; R_1, which released fMet tRNA in the presence of UAG and UAA but not of UGA; and R_2, which released in the presence of UAA and UGA, but not of UAG.

CASKEY et al. (1968) discuss the theory that *R* apparently contains an "anticodon" (in a rather broad sense that would perhaps include a region of a protein) that recognizes UAA, UAG and UGA. Two possibilities are advanced, first that *R* contains three recognition sites, one for each of the three codons; second that there is one recognition site which interacts with U in the first position of the codon *plus* an interaction with A or G in the second or third positions of the chain-terminating codon but not with G in both positions. Evidently *R* is subject to displacement by suppressor tRNA molecules, for suppressor strains can insert amino-acids at the sites of chain-terminating codons. It has been noted, however, that the so-called ochre suppressors (UAA suppressors), as compared with amber suppressors (UAG suppressors) are only weakly effective in repairing the gap in a protein produced by a point mutation from an amino acid codon to UAA. Perhaps R_1 has a stronger affinity for UAA than it does for UAG, and therefore R_1 is not readily displaced from UAA by a suppressor tRNA with the anticodon UUA.

Is the Code Universal?

A scientific purist might answer this question by saying that this cannot be decided until a majority of living species has been examined. Let us approach the question in another manner: What is the implication of the finding that the code is essentially the same in widely-differing organisms? Consider the case of yeast and *E. coli*. The two organisms differ from each other with respect to the various components of the protein synthesizing system, including differences in the base sequences and lengths of tRNAs for the same amino acid, in the base sequences of ribosomal RNAs (SUGIURA and TAKA-

NAMI, 1967) and in the number and kinds of modified bases in the tRNA molecules. In addition, it is safe to conclude that the primary structures of the proteins of yeast differ from any homologous counterparts in *E. coli*, including, of course, the aminoacyl tRNA synthetases (DOCTOR et al., 1966). This conclusion is reached by noting that, with rare exceptions, such as histones, it is only in closely related species that two homologous proteins or polypeptides have been found to be identical, such as human and chimpanzee hemoglobins A; bovine, sheep and porcine cytochromes *c*; and sheep and goat fibrinopeptides A. Despite the differences between yeast and *E. coli* proteins, it is assumed that there is no anticodon that specifies different amino acids in yeast and *E. coli*, even though this inference is exemplified only by a single case: GΨA and GUA are anticodons for tyrosine in yeast and *E. coli* respectively. Support from comparisons of other species is afforded by the findings that IGA is a serine anticodon in both yeast and in the rat, and GmAA is a phenylalanine anticodon in both yeast and wheat.

It is assumed that codon-anticodon pairing is the same in all species, following the pattern shown in Table 3. It is quite possible that not all the anticodons are present in each species, but it seems certain that messenger RNA molecules in each species contain all 64 codons because of considerations of statistics and probability. Each species must therefore contain enough anticodons to translate all 61 codons for the twenty amino acids and must also provide for chain termination. The three codons that are known to signal chain termination are UAA, UAG and UGA. However, NIRENBERG et al. (1966) have suggested that UGA may possibly code for cysteine in the guinea pig. With this possible, and doubtful, exception, all experiments on amino acid incorporation have shown the same codon-amino acid relationships.

Given the above assumptions, it follows that constancy in the code is maintained during evolution by preservation of the anticodon-amino-acid relationship. This is a function of tRNA molecules and their activating enzymes, the aminoacyl tRNA synthetases. Mutations that change an anticodon will be lethal if they change it so that it pairs with the codon for a different amino acid. Similarly, mutations in the amino acid recognition site of a tRNA molecule must not be such that they will change it to pair with the aminoacyl tRNA synthetase for a different amino acid, or "wrong" amino acids will be inserted throughout the proteins of the organism at many, perhaps all, of the sites that are normally occupied by the "right" amino acid.

Other regions of tRNA molecules are evidently subject to changes that can be presumed not to have any effect upon function. For example, it seems probable that the differences between two yeast serine tRNAs (Table 7), consisting of one U→C and two G→A interchanges, are neutral, i.e., nonfunctional, substitutions.

The Code and Chain Initiation

By what seems a fortunate accident, synthetic polyribonucleotides of widely-varying base composition and sequence can attach themselves to ribo-

somes *in vitro* and can function as messengers for synthesis of polypeptides, without the necessity for a specific base sequence to initiate the process of translation. The initiation process *in vivo* is evidently much more restricted in terms of the genetic code. Methionine, in its N-formyl modification, is an amino acid that has been identified as the initiator of polypeptide chains. It is difficult to decide whether other amino acids can exercise the same function, because the N-terminal formyl-methionine residue is known to be removed *in vivo* following completion of the synthesis of certain protein molecules in *E. coli* cells, and this raises the possibility that other "initiators" might be similarly removed.

The current interpretation of the role of the code in polypeptide chain initiation is as follows: The codon AUG can start chains by pairing with formylmethionyl tRNA, which has the identified anticodon CAU. This anticodon may also pair with GUG, and, weakly, with GUA as indicated by experiments with synthetic polyribonucleotides having the sequences $(UG)_n$ and $(GUA)_n$ respectively (GHOSH et al., 1967). The trinucleotides UUG and CUG have been reported to bind with formylmethionine tRNA, but there is no evidence that they can serve as chain initiators.

Experiments with synthetic polyribonucleotides of known sequence indicate that the initiator codon AUG need not necessarily occupy the left-hand terminus of the chain, but that it can be preceded by two or three nucleotides, such as AA or AAA, which are ignored, so that the polypeptide starts with formylmethionine rather than with asparagine (AAU) or lysine (AAA).

The initiation of polypeptide chains with N-formylmethionine is mediated by a special molecule of tRNA, formylmethionine tRNA (Met–$tRNA_f$). This molecule has the distinctive property of bringing about the enzymatic formylation of methionine attached to the tRNA. The formyl group is derived from the conventional carrier of such groups, 10-formyl tetrahydrofolic acid. The step of formylation takes place after the methionine molecule has been attached to Met–$tRNA_f$. Evidently the formylation reaction is brought about by an enzyme which recognizes the tRNA molecule. Met–$tRNA_f$ has, however, the anticodon CAU which is presumably identical with the anticodon in the tRNA which conveys unformylated methionine to internal sites in polypeptide chains. Only one anticodon, CAU, is possible for methionine according to present concepts, because other anticodons recognizing AUG (IAU, UAU) would be ambiguous for isoleucine. When formylated, Met–$tRNA_f$ is written as F–Met–$tRNA_f$.

F–Met–$tRNA_f$ was found to recognize the valine codon GUG at the 5′-terminus of ribopolynucleotides with the alternating sequence GUGUGU... so that a polypeptide starting with N-formylmethionine and continuing with alternate residues of cysteine and valine was synthesized (GHOSH et al., 1967).

It is accepted that there are two sites on the ribosome to which tRNA molecules may be bound. The first of these is the *polypeptide site*, so termed because it is occupied during peptide synthesis by the tRNA to which is attached the growing polypeptide chains. The adjoining *amino acid site*

becomes occupied by an incoming aminoacyl tRNA which will become attached to the peptidyl tRNA by the formation of a peptide linkage and the consequent release of the tRNA to which the peptide has been attached. Prior to the initiation of protein synthesis, both sites on the ribosome must evidently be vacant. It is at this point that Met–$tRNA_f$ becomes bound to the ribosome in response to the initiation codon AUG at, or near, the beginning of a cistron which has been transcribed into the messenger. The initial site to be occupied on the ribosome is the polypeptide site.

The formylation of the NH_2 group of methionine seems a logical step in the initiation of polypeptide chains, because it places a "peptide-like" linkage on the primary amino group, and thus sets the stage for the attack of the α-carboxyl group by the incoming $-NH_3^+$ group of the next amino acid, just as occurs repeatedly in the succeeding steps of chain elongation. It perhaps seems paradoxical that unformylated Met–$tRNA_f$ can serve for the initiation of polypeptide chains (CLARK and MARCKER, 1966). The effect of Met–$tRNA_f$ in initiation was increased by formylation, but the findings seem to indicate that the configuration of the Met–$tRNA_f$ molecule, rather than the formylation of methionine, is the predominating factor in the selection of this tRNA by the initiation process.

The observation poses the question: Can unformylated Met–$tRNA_f$ carry methionine to internal or 3′-terminal AUG codons? This was explored by ONO et al. (1968), who found that the "polymerization factors" S_1 and S_3 formed complexes with most (and possibly all) aminoacyl tRNAs *except* Met–$tRNA_f$ and F–Met–$tRNA_f$. The complexing step was thought to be a necessary preliminary to the attachment of the aminoacyl tRNA to the internal or terminal codons in the messenger RNA-ribosome complex. Factors S_1, S_2 and S_3 apparently are components of the translocation factor ("T factor"; LUCAS-LENARD and LIPMANN, 1966; LIPMANN, 1969).

The initiation of polypeptide synthesis involves magnesium ion, guanosine triphosphate (GTP) and several protein initiation factors (F_1, F_2, F_3 and F_4) which are attached to ribosomes but are easily removed *in vitro*. N-formyl methionyl $tRNA_f$ is bound to a 30S ribosomal subunit by an initiator codon, following which a 50S ribosomal unit becomes attached to the 30S subunit, and a 70S ribosome is thus formed. A second aminoacyl tRNA then becomes bound to the next codon in the strand of mRNA and a peptide bond is formed between the two amino acid residues. The binding requires GTP and a protein termed the T factor. Moving the 70S ribosome along the mRNA molecule needs GTP and a protein in the supernatant fraction of cell extracts, the G factor. A detailed description of the intricacies that have so far been discovered in the translation process is beyond the scope of this article, which will be confined to the coding aspects.

The initiation of polypeptide synthesis in cell-free systems with synthetic polyribonucleotides as messengers has been studied by several investigators, including WAHBA et al. (1966), SALAS et al. (1967) and THACH et al. (1966). The findings may be summarized as follows: The initiation factors F_1, F_2 and F_3

are necessary for starting polypeptide chains with phage RNA as messengers *in vitro* but not with synthetic polynucleotides. However, the presence of AUG as the beginning sequence of synthetic ribonucleotides greatly increased the incorporation of amino acids; for example, lysine incorporation was 10-fold greater with $AUGA_{18}$ than with A_4CA_{19} (SALAS et al., 1967). Similarly phenylalanine incorporation with $AUGU_{20}$, $AAUGU_{20}$, $AAAUGU_{20}$ or $AAAAUGU_{20}$ was 5-fold or more greater than with $ACGU_{20}$, $AACGU_{20}$, $AAACGU_{20}$ or $AAAACGU_{20}$ (THACH et al., 1966), leading to the conclusion that AUG acted as a "phasing" codon even when preceded by one to three additional A residues.

The RNA molecules synthesized *in vitro* on templates consisting of the replicative form of fd phage contained triphosphate groups attached to the 5'-ends (SUGIURA et al., 1969). Whether, preceding translation, such triphosphate groups participate in the attachment of "natural" mRNA molecules to the 30S ribosomal subunit is not known.

The processes of initiation and termination of polypeptide chains make it of much interest to examine the beginning and ending sequences of mRNA molecules, or of molecules of viral RNA, since these function as mRNA, i.e., as templates for polypeptide synthesis. Viral RNA molecules, however, have a dual function because they must function as templates for the synthesis of a complementary molecule: the minus strand of the replicative form (double-stranded) of the viral RNA. Consequently, we should expect the beginning and end of the plus strand to be occupied by sequences which signal the attachment and release of the viral RNA polymerase (replicase) enzyme, and not necessarily the initiation and termination codons for polypeptide synthesis.

Beginning Sequences

RNA molecules probably start in most cases with a purine, because of a preference on the part of RNA polymerase for starting transcription at a pyrimidine on the template (MAITRA and HURWITZ, 1965). SZYBALSKI et al. (1966) found that C-rich regions in DNA serve as initiation points for transcription. Most of the known RNA molecules start with G. The starting nucleotide in an RNA molecule retains its 5'-triphosphate group when the reaction is carried out *in vitro* with RNA polymerase. Triphosphates precede the polynucleotide chain in several viral RNAs as first shown by TAKANAMI (1966) and in three messenger RNA molecules where 5'-termini have been identified (SUGIURA et al., 1969). TMV RNA, however, does not have a triphosphate group at the 5'-terminus, nor do the transfer RNAs or ribosomal RNAs that have been examined.

End Sequences (3'-terminal or Right-hand End Sequences)

The final nucleotide in RNA molecules has a free 3'-OH group. All the viral RNAs that have so far been examined end with $-CCCA_{OH}$ (Table 7) and none of them has a chain-terminating codon in the end regions that have

so far been identified. Since tRNA molecules always end with $-CCA_{OH}$, which is added by a special enzyme after the RNA has left its DNA template, it is suspected that the same process takes place with viral RNA. This end serves as the beginning of the template during the synthesis of the minus strand

Table 7. *5′ Termini and 3′ Termini of mRNA and viral RNA molecules*

Molecule	5′ Termini	Reference
Messenger RNAs of fd phage	pppAUG ... pppGUA ... pppGUU ...	SUGIURA, OKAMOTO and TAKANAMI (1969)
TMV	C, G, A or U	MANDELES (unpublished)
f2, R17	pppG ...	DAHLBERG (1968), ROBLIN (1968)
MS2	pppGGGU ...	DE WACHTER et al. (1968),
MS2	pppGGU ...	GLITZ (1968)
$Q\beta$	pppGGGGAAC ...	DE WACHTER and FIERS
$Q\beta$	pppGGGGCAAC ...	(1969)
	3′ Termini	
TMV	... $GCCCA_{OH}$	MANDELES (1967)
f2, R17, MS2	... $GUUACCACCCA_{OH}$	DAHLBERG (1968), DE WACHTER and FIERS (1967), WEITH and GILHAM (1967)
$Q\beta$	... $GCCCUCCUCUCUCCCA_{OH}$	DAHLBERG (1968)

when the replicative form (double-stranded) of the viral RNA is synthesized. The region may therefore function as a recognition site for replicase, and the termination of translation may occur at a point some distance to the left of the $-CCA_{OH}$. The evidence for termination of polypeptide chains by UAG, UAA and UGA depends on experiments with synthetic polynucleotides and with mutations that produce chain-terminating codons in internal regions of DNA or RNA molecules. It is evident that the terminal –CCA group in TMV, which is a code for proline and not for polypeptide chain termination, does not code for the terminal amino acids of the coat proteins of these viruses, which end with threonine and tyrosine.

Transfer RNA

Transfer RNA stands at the center of most problems regarding the code. The announcement of the primary structure of a yeast alanine tRNA by HOLLEY and co-workers (1965) included three proposals for possible two-dimensional structures. These had regions of helicity, produced by G · C and A · U pairing, alternating with single-stranded regions. Of these three proposals, the "clover-leaf" formula (Fig. 1) has gained wide acceptance because

the primary structures of all tRNAs whose composition is known may be superimposed on it. A proposal for a three-dimensional structure corresponding to the clover-leaf formula was made recently by CRAMER et al. (1968).

HOLLEY et al. proposed that the anticodon, pairing with GCN, could be between two hU residues in the sequence hU–C–G–G–hU or could be the sequence I–G–C. Studies with other tRNAs have established the anticodon as being in the middle of the bottom, or "anticodon", loop (Fig. 1). For example, in the mutation which converts normal tyrosine tRNA to tyrosine

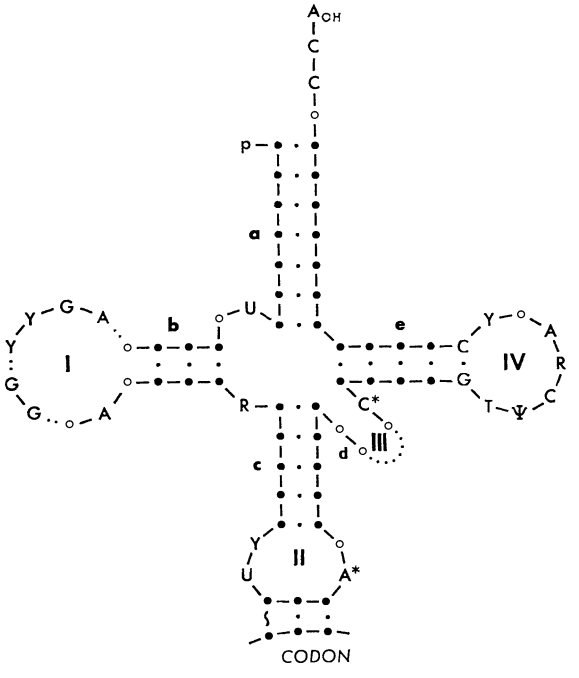


Fig. 1. Generalized two-dimensional "clover-leaf" formula for tRNA. Solid circles indicate bases in helical regions that are usually paired by hydrogen bonding; open circles, unpaired bases; a, b, c, d, e, helical regions; I, II, III, IV; "loops". The pairing at the bottom of the diagram is between anticodon and codon; ~, "wobble" pairing. Designated bases are invariant (see Table 8). *R* purine; *Y* pyrimidine. Gaps (see Table 8) are indicated by ···; * modified base; *T* ribothymidine; Ψ pseudouridine

suppressor tRNA in *E. coli*, this sequence changes from GUA to CUA, which pairs with the chain-terminating codon UAG (GOODMAN et al., 1968). Each anticodon is flanked by U, and A or modified A.

The number of different tRNAs has not been determined for any species of organism. A minimum of 31 is required to translate all the codons, in terms of the wobble hypothesis. Probably all organisms have at least this number of different tRNAs, and many organisms have undoubtedly a considerably greater number. Duplication of genes for tRNA seems to occur frequently. Two serine tRNAs from yeast have been identified which differ from each other in only three residues.

Any speculation on the evolution of the genetic code must include a detailed consideration of the evolution of tRNAs. These molecules are subject to mutation and evolutionary differentiation resulting from their origin by transcription of DNA sequences, since all such sequences undergo genetic changes including base replacements, and shortening and lengthening by deletions and recombinations.

The Amino-Acid Recognition Site in tRNA

The charging of a tRNA molecule with an amino acid takes place by means of esterification of the α-carboxyl group of the amino acid with the 2′- or 3′-hydroxyl group of ribose in the right-hand terminal adenosine component of tRNA. The amino acid in this reaction is in the form of an adenylate which is bound to an enzyme, an *aminoacyl tRNA synthetase*. This enzyme recognizes a site on a tRNA molecule which is specific for the particular amino acid carried by the synthetase. This is termed the *recognition site*. It is not identical with the anticodon. Various attempts have been made to identify the recognition site. The following matters must be considered:

(i) A mutation can take place in the anticodon without changing the specificity of the tRNA molecule. This was shown by determining the structure of an *E. coli* tyrosine suppressor tRNA, which has the anticodon CUA, specific for UAG (chain termination).

(ii) If two tRNAs for different amino acids are present in the same organism, the recognition site must be in some portion of the molecule which is not identical in the two tRNAs. The primary structure of twelve different tRNAs is shown in Table 8. Five of these are from yeast. Many of the nucleotides are identical in at least two of them. The recognition site cannot be composed entirely of any of these nucleotides. This proviso appears to exclude the seven-nucleotide loop that starts with TΨC, because valine and isoleucine *Torulopsis* tRNAs are identical in this region, and indeed, in the three base pairs that adjoin the loop. The hU loops in these two tRNAs are almost identical, also.

(iii) If two tRNAs for the same amino acid from different organisms can both be charged by the same aminoacyl synthetase, there should be identical recognition sites in the two tRNAs. Conversely, if the enzyme from organism 1 will *not* charge the tRNA from organism 2, the two recognition sites must differ.

A proposal was made (Dudock et al., 1968) that the recognition site was in the dihydrouridine loop. The dihydrouridine loops are very different for yeast and *E. coli* tyrosine tRNAs as follows:

Yeast –A–G–hU–hU–Gm–G–hU–hU–hU–A–
E. coli –A–G– –C –Gm–C–C –C –A –A–

but the two anticodon loops are quite similar, as follows:

Yeast –C–U–G–Ψ–A–m_2A–A–
E. coli –C–U–G–U–A–A† –A–

Table 8. *Base sequences of yeast alanine, E. coli formylmethionine, E. coli and yeast valine, Torulopsis isoleucine, yeast and rat serine, yeast and E. coli tyrosine, yeast and wheat phenylalanine, and E. coli methionine transfer RNAs*

	Ala	F Met	Val		Ile	Ser		Tyr		Phe		Met
	Y	EC	EC	Y	TY	Y	R	Y	EC	Y	W	EC
1	pG	pC	pG	pG	pG	pG	pG	pC	pG	pG	pG	pG
	G	G	G	G	G	G	U	U	G	C	C	G
	G	C	G	U	U	C	A	C	U	G	G	C
	C	G	U	U	C	A	G	U	G	G	G	U
	G	G	G	U	C	A	U	C	G	A	G	A
	U	G	A	C	C	C	C	G	G	U	G	C
	G	G	U	G	U	U	G	G	G	U	A	G
	*U	U†	Ut	U	U	U	U	U	Ut	U	U	U
	mG	G	A	mG	G	G	G	A	Ut	A	A	A
10	G	G	G	G	G	G	G	mG	C	mG	mG	G
	C	A	C	U	C	C	C	C	C	C	C	C
	G	G	U	C	C	acC	acC	C	C	U	U	U
	C	C	C	Ψ	C	G	G	A	G	C	C	C
	G	—	—	—	—	—	—	—	—	—	—	—
	U	—	—	—	—	—	—	—	—	—	—	—
	*A	A	A	A	A	A	A	A	A	A	A	A
	*G	G	G	G	G	G	G	G	G	G	G	G
	hU	C	C	hU	hU	hU	hU	hU	C	hU	hU	U
	C	C	hU	C(hU)	hU	—	—	hU	—	hU	hU	hU
20	—	U	—	—	—	—	—	—	—	—	—	—
	*G	G	G	G	G	Gm	Gm	Gm	Gm	G	G	G
	*G	G	G	G	G	G	G	G	G	G	G	G
	—	—	—	hU	hU	hU	hU	hU	C	—	—	hU
	—	—	—	—	—	—	—	hU	C	—	—	—

Table 8 (Continued)

	Ala	F	Val		Ile	Ser		Tyr		Phe		Met
	Y	Met			TY							EC
		EC	EC	Y		Y	R	Y	EC	Y	W	
	hU	hU	G	hU(C)	hU	hU	hU	hU	A	G	G	hU
	*A	A	A	A	A	A	A	A	A	A	A	A
	G	G	G	U	A	A	A	A	A	G	G	G
	C	C	A	G	G	G	G	G	G	A	A	A
	G	U	G	G	G	G	G	G	G	G	G	G
	C	C	C	C	C	C	C	C	G	C	C	C
	m_2G	G	A	A	m_2G	m_2G	m_2G	m_2G	A	m_2G	m_2G	A
32	C	U	C	Ψ	Ψ	A	A	C	G	C	Ψ	C
	U	C	C	C	G	A	Ψ	A	C	C	C	A
	C	G	U	U	G	A	G	A	A	A	A	U
	C	G	C	G	U	G	G	G	G	G	G	C
	C	G	C	C	G	A	A	A	A	A	A	A
	U	Cm	C	Ψ	C	Ψ	mC	C	C	Cm	Cm	C
	*U	U	U	U	U	U	U	U	U	U	U	U
	I	C	N	I	I	I	I	G	G†(C)	Gm	Gm	C†
40	G	A	A	A	A	G	G	Ψ	U	A	A	A
	C	U	C	C	U	A	A	A	A	A	A	U
	*mI	A	mA	A†(A)	A†	iA	iA	iA	A†	N	N	A†
	Ψ	A	A	C	A	A	A	A	A	A	A	A
	G	C	G	G	C	Ψ	Ψm	Ψ	Ψ	Ψ	Ψ	Ψ
	G	C	G	C	G	C	C	C	C	mC	C	G
	G	C	A	A	C	U	C	U	U	U	U	A
	A	G	G	G	C	U	A	U	G	G	G	U
	G	A	G	A	A	U	U	G	C	G	A	G
	A	A	G	A	A	Um	Um	A	—	A	A	G
50	G	G	G	C	G	G	G	G	C	G	G	G

Table 8 (Continued)

	Ala	F Met	Val		Ile	Ser		Tyr		Phe		Met
	Y	EC	EC	Y	TY	Y	R	Y	EC	Y	W	EC
	—	mG(A)	m_7G	—	A	G	G	A	G	mG	mG	mG
	—	—	—	—	—	G	G	—	U	—	—	
	—	—	—	—	—	C	G	—	C	—	—	—
	—	—	—	—	—	U	U	—	A	—	—	—
	—	—	—	—	—	U(C)	mC	—	C(U)	—	—	—
	—	—	—	—	—	U	U	—	A(C)	—	—	—
	—	—	—	—	—	G	C	—	G	—	—	—
	—	—	—	—	—	C	C	—	A	—	—	—
	—	—	—	—	—	C	C	—	C	—	—	—
60	—	—	—	—	—	C	C	—	U	—	—	—
	U†	U	U	hU(—)	hU	G	G	hU	U	U	hU	N
	*C	C	C	mC	mC	mC	mC	mC	C	C	C	C
63	U	G	G	C	A	G	G	G	G	mC	G	A
	C	U	G	C	G	C	C	G	A	U	C	C
	C	C	C	C	C	A	A	G	A	G	G	A
	G	G	G	A	A	G	G	C	G	U	U	G
	*G	G	G	G	G	G	G	G	G	G	G	G
	*T	T	T	T	T	T	T	T	T	T	T	T
	*Ψ	Ψ	Ψ	Ψ	Ψ	Ψ	Ψ	Ψ	Ψ	Ψ	Ψ	Ψ
	*C	C	C	C	C	C	C	C	C	C	C	C
71	G	A	G	G	G	G(A)	G	G	G	G	G	G
	*A	A	A	mA	mA	A	mA	mA	A	mA	mA	A
	U	A	U	U	U	G(A)	A	C	A	U	U	A
	U	U	C	C	C	U	U	U	U	C	C	U
	*C	C	C	C	C	C	C	C	C	C	C	C
	C	C	C	U	U	C	C	G	C	A	A	C
	G	G	G	G	G	U	U	C	U	C	C	C

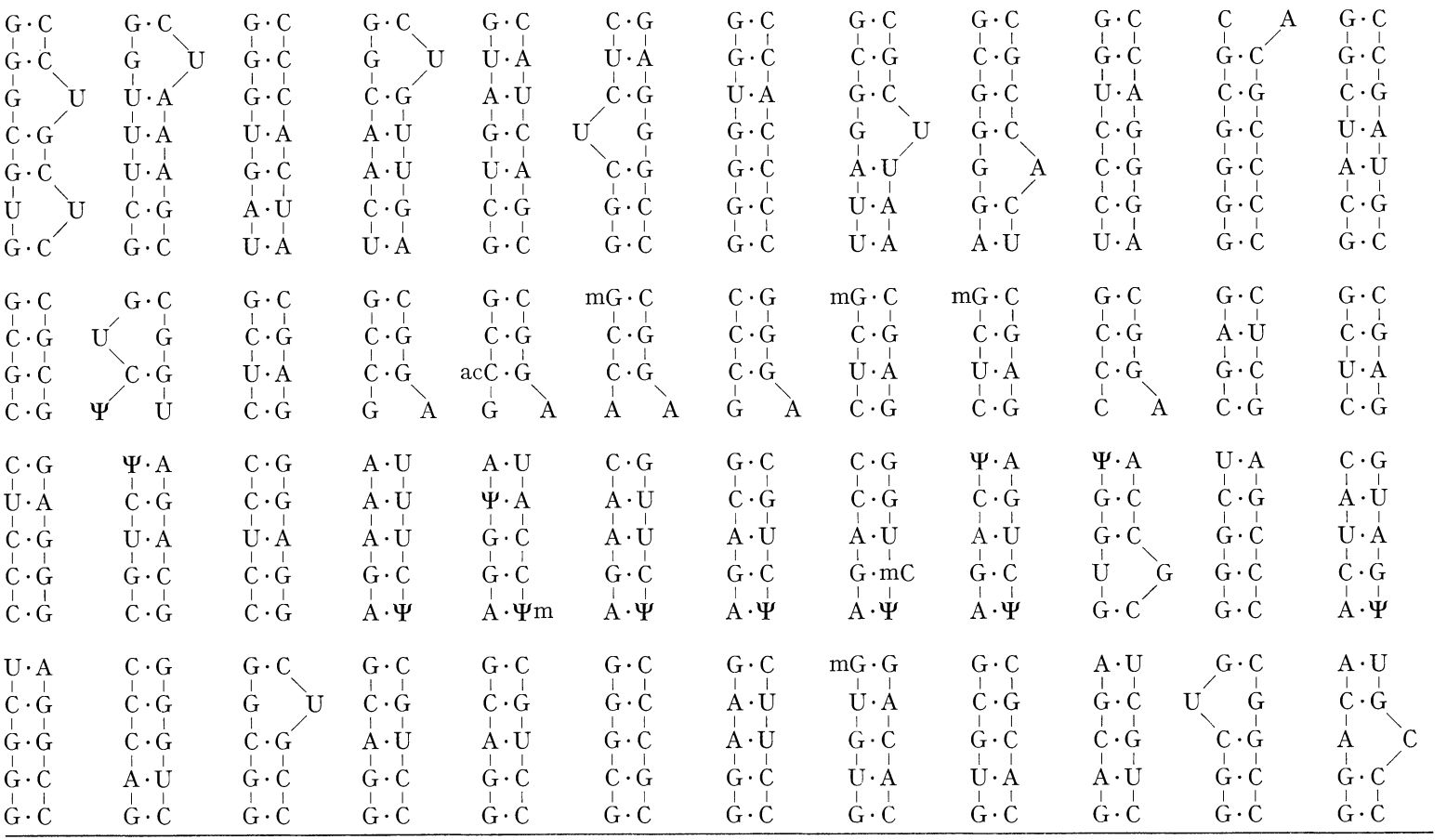
Table 8 (Continued)

	Ala Y	F Met EC	Val EC	Val Y	Ile TY	Ser Y	Ser R	Tyr Y	Tyr EC	Phe Y	Phe W	Met EC
	G	G	U	G	C	G	G	C	U	A	G	G
	A	C	C	G	U	C	C	C	C	G	C	U
80	C	C	A	C	A	A	C	C	C	A	U	C
	U	C	U	G	G	G	G	C	C	A	C	G
	C	C	C	A	G	U	A	G	C	U	A	U
	G	C	A	A	G	U	C	G	C (A)	U	C	A
	U	G	C	A	A	G	U	G	A(C)	C	C	G
	C	C	C	U	C	U	A	A	C	G	G	C
	C	A	C	C	C	C	C	G	C	C	C	C
	A	A	A	A(—)	A	G C C A—OH	G	A	A(C)	A	A	A

Abbreviations. Y = yeast; EC = *E. coli;* W = wheat; N = unspecified nucleoside; hU = dihydrouridine; Ut = 4-thiouridine; Cm = O-methyl cytidine; Gm = O-methyl guanosine; mG = methyl guanosine; m^7G = 7-methyl guanosine; mA = methyladenosine; Ψ = pseudouridine; m_2G = dimethylguanosine; mI = methyl inosine; m_2A = dimethyl adenosine; m_2C = dimethyl cytidine; iA = isopentenyl adenine; acC = acetylcytidine; mC = methylcytidine; † = modified nucleoside; * = designates nucleosides that are identical in all the tRNAs listed; lines separate the helical regions; hyphens indicate evolutionary "gaps" (CANTOR, 1968). Letters in parentheses are residues in variants of the tRNAs beside which they appear; those beside the yeast valine sequence are differences that are present in *Torulopsis utilis* valine tRNA.

Sources. HOLLEY et al., 1965; ZACHAU et al., 1966; MADISON et al., 1966; RAJ BHANDARY et al., 1967; BAYEV et al., 1967; TAKEMURA et al., 1968; GOODMAN et al., 1968; DUBE et al., 1968; DUDOCK et al., 1968, 1969; NISHIMURA et al., 1969; STAEHELIN et al., 1968; CORY et al., 1968; YANIV and BARRELL, 1969.

Table 9. *Helical regions of tRNAs of known sequence*



Yeast and *E. coli* tyrosine tRNAs may therefore be used for comparing the effects of tyrosine tRNA aminoacyl synthetase from one of the respective organisms on the tRNA from the other organism. DOCTOR et al. (1966) found that there was absolute species-specificity. The *E. coli* enzyme would not charge yeast tyrosine tRNA 1 and the yeast enzyme would not charge either of two tyrosine tRNAs obtained from *E. coli* as follows:

Source of tRNA		Source of enzymes	
		E. coli	Yeast
Tyr I	Yeast	—	1.37
Tyr I	*E. coli*	1.08	—
Tyr II	*E. coli*	1.33	—

The figures are for mμmoles of tyrosine incorporated per unit of tRNA, as calculated from ultraviolet absorption.

In contrast, when a similar experiment was carried out with yeast and wheat germ phenylalanine tRNAs by DUDOCK et al. (1968), they found that either enzyme would charge either tRNA. The dihydrouridine loops of these two tRNA are identical, as follows:

$$-A-G-hU-hU-G-G-G-A-$$

but the other regions of the two molecules differ from each other. DUDOCK et al. (1968) suggested that the dihydrouridine loop identifies the tRNA to the activating enzyme. A different proposal was made by SCHULMAN and CHAMBERS (1968), who studied the inactivation of alanine tRNA by ultraviolet light, and concluded that the target for inactivation was located in the first three nucleotide pairs of the top helix (Table 9). These are identical in yeast and wheat phenylalanine tRNAs. However, *E. coli* F–Met and Met tRNAs are both charged by the same enzyme (HARTLEY et al., 1969) but differ from each other in the first three nucleotide pairs (Table 9), and in the dihydrouridine loops (Table 8).

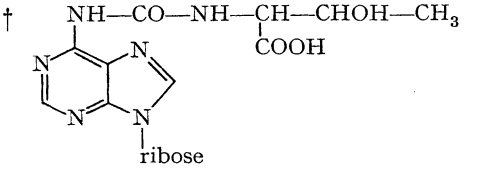
The Role of the Modified Base at Position 42 (Table 8) in Codon-anticodon Pairing

The base at position 42 is A or modified A in all the tRNAs in which it has been identified. Certain side chains attached to this adenosine residue are remarkable for their complexity and for the fact that they have not been detected elsewhere in tRNA or other RNA molecules. The intriguing possibility that these unusual side-chains are involved in codon-anticodon pairing was pointed out by NISHIMURA et al. (1969). The *E. coli* tRNAs pairing with codons starting with U have 2-methylthio N^6-(Δ^2-isopentenyl) adenosine in position 42 in all cases in which this side chain and its location have been identified. The corresponding yeast and rat adenosines lack the methylthio radicle in the side chain (Table 10), but contain the N^6-isopentenyl side chain.

Table 10. *Relation between side chains at tRNA position 36 and base at first position of certain codons*

Side chain on adenosine residue. Known (*) or assumed to be at position 42	Transfer RNA source	amino acid	Codons
a) 2-methylthio-N^6-(Δ^2-isopentenyl)	*E. coli*	Phe	UUY
	E. coli	Ser	UCN
	E. coli	Tyr*	UAY
	E. coli	Leu	UUR
	E. coli	Trp	UGG
	E. coli	Cys	UGY
b) N^6-(Δ^2-isopentenyl)	yeast, rat	Ser*	UCN
	yeast	Tyr*	UAY
c) *N*(9-β-D-ribofuranosylpurin-6-ylcarbamoyl)threonine†	*Torulopsis*	Ile*	AUY, AUA
	E. coli	Ser III	AGY
d) 6-methyl	*E. coli*	Val	GUN
e) 1-methyl (on inosine)	yeast	Ala	GCN
f) No side-chain	*E. coli*	F-Met	AUG, GUG
	Torulopsis	Val	GUN
g) Unidentified side-chain	yeast	F-Met	AUG, GUG
	yeast	Val	GUN

Side-chain unidentified, but differing from (a): *E. coli*; Asp(GAY); His(CAY); Leu(CUN); Met(AUG).

† 

Source: NISHIMURA et al. (1969), TAKEMURA et al. (1968, 1969), RAJ BHANDARY and GHOSH (1969), HOLLEY et al. (1965).

Furthermore, both these modified adenosines have cytokinin activity (HALL et al., 1967) and this activity is present in cysteine tRNA (BOCK, cited by NISHIMURA et al., 1969). In contrast, *E. coli* tRNAs for the following amino acids were found *not* to contain 2-methylthio N^6-(Δ^2-isopentenyl) adenosine: serine III (which pairs with AGC), aspartic acid, histidine, valine, leucine, methionine and formyl-methionine. These amino acids are, or can be, coded by codons starting respectively with A; A; C; G; C; A; and A or G.

Apparently the adenosine in position 42 has a specific role in codon-anticodon pairing, and an unusual side-chain may be involved in this function. These findings add a new complexity to the coding mechanism and emphasize its sophisticated character. Tentatively, the following suggestions are made with respect to codon-anticodon pairing:

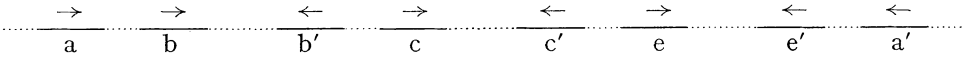
(i) The base in tRNA position 38 is unmodified U, which leaves position 39 free to "wobble".

(ii) Watson-Crick pairing between codon and anticodon is aided by special side-chains on A at position 42.

(iii) There are preliminary indications that the various codon-anticodon base pairs at position 41 are reinforced by specific side-chains at position 42. For example, the U · A base pair appears to be reinforced by an isopentenyl side-chain, and the A · U base pair by *N*(9-β-D-ribofuranosyl-purin-6-yl-carbamoyl) threonine, identified by SCHWEIZER et al. (1968).

The Evolution of Transfer RNA

The problem of the evolution of transfer RNA poses the following difficulty: How did the molecule evolve so that about 60% of it consists of sequences that exhibit reverse complementarity to other sequences elsewhere in the nucleotide chain? The pattern of complementarity is as follows:



The arrows indicate the direction of complementarity rather than polarity, and the numbers reflect the helical regions as shown in Fig. 1, with which the above diagram should be compared.

A suggested evolutionary pathway is in Fig. 2. The base sequences are imaginary, and have been inserted only to indicate complementarity and polarity. For convenience in depiction, the length has been kept shorter than that of actual tRNA. The first step is duplication of a piece of DNA of the requisite length. The two duplicate pieces then encounter each other after one has turned end-for-end, and become joined as shown in Fig. 2. Transcription of the resulting piece of DNA by RNA polymerase produces a single strand of RNA complementary to one of the two strands of DNA. This can fold back on itself (a) to form a helical double-stranded RNA molecule. The usual process of evolutionary differentiation takes place by point mutations occurring in the DNA. A loop (b) is formed at the bottom of the RNA molecule, resulting from loss of complementarity occasioned by point mutations, and, as a result, a primitive single-stemmed tRNA molecule is formed with an anticodon, shown as CGU, at the bottom of the loop. As differentiation continues (c), further looping out takes place (d), until, eventually, the familiar clover-leaf structure with two additional loops and two additional helical regions, is formed (e). This duplicates repeatedly; the anticodon regions differentiate, and so do the recognition sites for amino acids until a family of tRNA molecules appears.

The primary structures of twelve identified tRNA (Table 8) have an evolutionary homology that is quite similar to the case of the cytochromes *c*. In both cases, there are considerable numbers of invariant residues that are identical in all the members of the family that have been examined. In the case of the cytochromes *c*, there are 35 identical amino acid residues in about 30 cytochromes *c* that have been examined; however, many of the 30 are identical to each other in considerably more than 35 residues because of close phylogenetic relationships. The number of actually invariable residues has been estimated as 27 to 29 (FITCH and MARGOLIASH, 1967; JUKES and CANTOR,

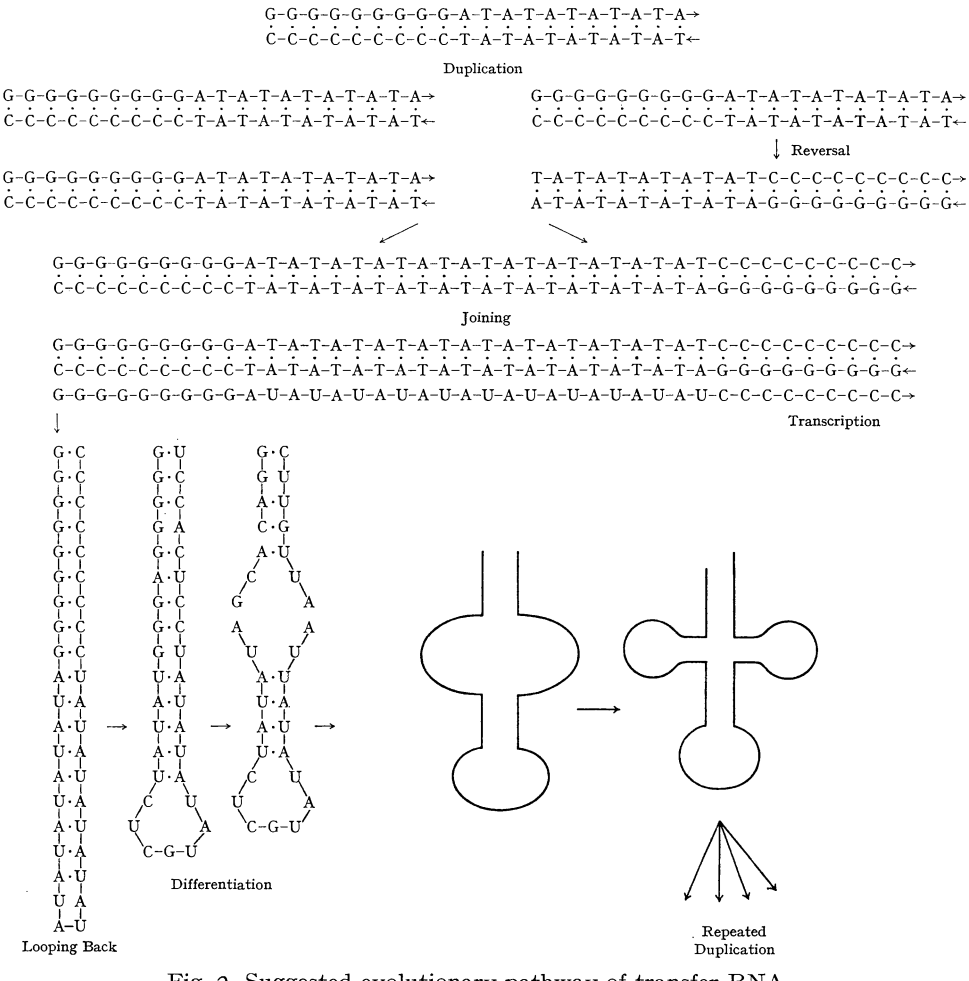


Fig. 2. Suggested evolutionary pathway of transfer RNA

1969) in sequences that range from 103 to 112 residues in length. The content of invariant amino acids is therefore about 26%.

The tRNAs in Table 8 have 15 identical residues which correspond to about 20% of the molecules, since they have an average content of about 77 nucleotides per molecule. The tRNA sequences vary in length, apparently as a result of evolutionary deletions and additions, especially in the hU loop and in the region connecting the third and fourth helices. The first, third and fourth helical regions are constant in length, and contain respectively seven, five and five base pairs, except in the case of the first helical region of F–Met–$tRNA_f$ (Table 9).

The similarity of all the tRNAs in Table 8, the general structure shown in Fig. 1, and especially the presence of 15 invariable residues, strongly support the conclusion that all tRNAs have evolved from a single ancestral molecule by gene duplication and differentiation, just as in the case of the

cytochromes *c*. Such an ancestral molecule would not have transferred more than a single species of amino acid. Since functional proteins must contain several different amino acids, the conclusion is that the appearance of the ancestral "contemporary-type" tRNA must have been preceded by an earlier system of protein synthesis, long since extinct, which supplied the mechanism necessary for synthesizing a tRNA molecule of the type shown in Fig. 1.

The tRNA molecule has a quite sophisticated structure. If all the existing tRNAs are indeed descended from it, we must conclude that an earlier form of life existed which was sufficiently complex to produce such a molecule, and also to give rise to a family of related molecules that translated the genetic message into proteins which were the ancestors of those existing to-day.

There has been speculation on the composition of such proteins. It has been suggested (JUKES, 1965) that the present genetic code was preceded by a code in which all codons were of the general type XYN, where N is synonymously A, C, G or U. This inference is drawn from the fact that eight quartets of codons currently follow this pattern (Table 1). Conceivably these could represent the remnant of a version of the code in which all codons were assigned in this manner. In this case, a theory should explain the fact that when an amino acid is coded by two codons having the same two bases in positions 1 and 2, the two bases in position 3 are either U and C, or A and G, i.e., the pairs of codons for a single amino acid in Table 1 are never arranged in any of the following four groupings:

$$\begin{cases} XYU \\ XYA \end{cases} \quad \begin{cases} XYU \\ XYG \end{cases} \quad \begin{cases} XYC \\ XYA \end{cases} \quad \begin{cases} XYC \\ XYG \end{cases}$$

The theory should also explain the partition of the AUN codons between isoleucine and methionine, and of the UGN codons between cysteine, tryptophan and chain termination.

It was proposed that a definite sequence of changes involving tRNAs is responsible for a quartet of codons XYN becoming divided between two different amino acids (JUKES, 1967). The proposal depends on a redistribution that results from point mutations in tRNAs and aminoacyl synthetases. The first example is the isoleucine-methionine group of four codons. An isoleucine tRNA, obtained from *Torulopsis* yeast, has been completely identified and has the anticodon IAU (TAKEMURA et al., 1969). An *E. coli* methionine tRNA with the anticodon CAU has also been identified (CORY et al., 1968). The anticodon UAU shold not exist, except in suppressor mutations, because it would pair with codons, AUA and AUG, for two different amino acids, so that it presumably would be eliminated from wild types. It is proposed that an evolutionary change took place in the tRNA with the anticodon CAU that changed it from combining with isoleucine to combining with methionine. The aminoacyl synthetase that charged this tRNA presumably mutated so that it combined with methionine rather than with isoleucine. Perhaps this tRNA had evolved so that its recognition site differed from those in the

tRNAs having the anticodons IAU and GAU. This would lead to the existence of a specific isoleucyl synthetase for the "CAU tRNA" which would not charge the "IAU and GAU" tRNAs. The crucial mutation that changed this isoleucyl tRNA synthetase into methionyl tRNA synthetase added a new amino acid, methionine, to the chemistry of life.

Similarly the addition of tryptophan to the list of amino acids used in protein synthesis could have taken place by a change in the cysteinyl tRNA with the anticodon CCA so that this tRNA recognized tryptophan. In this case, however, it is assumed that the change took place at a time when the anticodon UCA did not exist. The anticodon ICA either did not exist or has disappeared, except in suppressor mutations which use the codon UGA.

We now examine the more drastic case exemplified by histidine and glutamine. It is necessary to assume that there were only two tRNAs that paired with the four codons in the CAN group; one with anticodon GUG, and the other with the anticodon UUG. These two tRNAs both transferred the same amino acid, but it is not possible to decide whether this was histidine or glutamine. Let us suppose that it was histidine. In this case, the "UUG tRNA" mutated so that it recognized glutamine. This mutation would assign the codons CAA and CAG to glutamine.

The final case to be considered is that of the four UAU codons, two of which, UAA and UAG, are signals for the termination of polypeptide chains. The only known anticodon pairing with any of the four is GUA, (or the equivalent GΨA and †GUA), present in tyrosine tRNA (Table 3a). The possibilities for the mechanism of chain termination include (i) the polypeptide chain, with its terminal peptidyl-tRNA ester linkage, becomes released from the ribosome, and is hydrolyzed by enzymes in the cytoplasm; (ii) special molecules exist which bind with chain-terminating codons and break the ester linkage, as discussed on p. 194.

The first of the two possibilities is currently the less attractive one, in view of the findings with the "release factor" (CAPECCHI, 1967; CASKEY et al., 1968). It is simpler from the evolutionary standpoint to presume that the codons UAA, UAG and UGA have never coded for amino acids.

It seems likely that the amino acid code is "frozen", i.e., that it is not subject to evolution. Changes in the amino-acid assignment of codons would produce a profound change in the composition and hence the function of the proteins of an organism, for all 64 codons are dispersed throughout messenger RNA molecules. A change in the assignments of any codon would almost certainly be lethal to any organism that lives to-day. We must assume that the organisms that existed during the evolution of the code were sufficiently simple to tolerate such changes. It is of interest to note that single amino acid substitutions corresponding to a number of the proposed evolutionary changes in the code are known in viable organisms, as follows:

Glutamine	$\rightleftharpoons$ Histidine	— β lactoglobulin
Glutamic acid	$\rightleftharpoons$ Aspartic acid	— TMV protein
Leucine	$\rightleftharpoons$ Phenylalanine	— TMV protein

Isoleucine	⇌ Methionine	— TMV protein
Asparagine	⇌ Lysine	— Hemoglobin, TMV protein
Cysteine	⇌ Tryptophan	— Yeast mitochondrial protein

How Old is the Code?

The codons, as defined in terms of base triplets, are as old as DNA and RNA, and are as simple as any other linear sequence of four variables. The translation process, in contrast, is highly complex and sophisticated. It includes ribosomes, various enzymes, and tRNA, as well as smaller molecules such as guanosine triphosphate. Of the larger molecular components, the one that is best defined in terms of present knowledge is tRNA.

As noted on page 210, the tRNAs appear to have evolved from a single archetypal molecule, as shown by the fact that 15 nucleotide residues are identical in homologous sites in all the tRNAs whose sequences are known. If the remaining portions of the molecules are compared, some striking facts emerge. The phenylalanine tRNAs of yeast and wheat (Table 8) are identical in 74 percent of the "variable" homologous sites. This is the same order of similarity as in the cytochromes *c* of these two organisms. Similarly, yeast and rat serine tRNAs are identical in 75 percent of their homologous sites. The tyrosine tRNAs of yeast and *E. coli*, by the same type of comparison, have 45 percent of identity and the valine tRNAs of yeast and *E. coli* have 48 percent. Since there is one chance in four that any two bases will be the same, the lowest average value for identity between two non-related sequences will be 25 percent. It seems likely, however, that in addition to the fifteen invariant residues in the tRNAs, there are other residues that show a tendency not to vary. Positions 1, 10, 11, 29, 30, 43, 50, 71 and 87 are constant in 10 or 11 of the 12 tRNAs in Table 8.

When the tRNAs for two *different* amino acids are compared in the same manner, the similarities range from 38 to 50 percent, with an average value of 41 percent. In these comparisons, the similarities are greater, on the average, for the *E. coli* tRNAs than for the yeast tRNAs. The most marked similarities between the tRNAs for different amino acids are as follows:

Comparison	*Percent similarity*
Val EC:Met EC	57
Val EC:Ala Y	54
Ala Y:FMet EC	52

The value of 41 % is not much less than the percentage of similarity between yeast and *E. coli* tRNAs for tyrosine (45 %) and valine (43 %). This finding may indicate that the present form of tRNA, as shown in Fig. 1 and Table 8, originated by duplication of an archetypal molecule of the general form shown in Fig. 1, not long before the evolutionary separation of bacteria and eukaryotic organisms, perhaps three billion years ago. The alternative interpretation is that a value of between 38 and 50 percent similarity between tRNAs, corresponding to a difference of 50 to 62 percent, represents an

evolutionary equilibrium which has been reached in the differentiation of the tRNAs of *E. coli* from those of higher organisms and of the tRNAs of different amino acids from each other.

We conclude that, prior to this, molecules of another type were used to transfer amino acids in protein synthesis. These molecules are now extinct.

The Relation of the Code to Evolutionary Replacements of Amino Acids

It is generally assumed that evolutionary replacements of amino acids in proteins are the result of point mutations which have become incorporated into the genome of the species. Such replacements are of wide occurrence, as may be seen by comparing homologous proteins. It appears that the changes are distributed randomly, and follow the Poisson distribution if the invariant residues are excluded from the calculation (JUKES, 1969). In this case, many codons must be changed more than once. In the differentiation of a protein such as a hemoglobin chain from its archetypal form, the number of replacements may correspond to as high as 80% of the residues in the molecule. In such a case, some codons must have undergone eight or more changes. This concept is seldom mentioned in articles on molecular evolution.

A point mutation can change a codon into any of nine other codons. A second mutation in the mutant codon can produce a change corresponding to a two-base change in the code, to a single-base change in the code (for example: GGU to GCU to GUU) or to a revertant to the original amino acid. The possibilities grow rapidly in number as the number of successive mutations increases, except that no change in the amino acid assignment of a codon may correspond to a change of more than three bases in the code. The subject was discussed by JUKES (1963). Many of the amino acid replacements that take place during the evolution of a protein are perforce lost in the record of the past which has been erased. It is noteworthy, however, that multiple changes at a single site are quite commonly found when groups of homologous proteins are examined. Nine different amino acids occupy an homologous site in various globins, and there are eight different amino acids at each of two sites in the cytochromes *c*.

Ambiguity and Miscoding

The term *ambiguity* will be used to refer to situations where a codon pairs with tRNAs for more than one amino acid. *Miscoding* refers to the incorporation of an amino acid in response to a codon which is assigned to a different amino acid in Table 1.

The question of ambiguity was raised and discussed by VON EHRENSTEIN (1966) as an explanation for the finding that preparations of rabbit hemoglobin did not have homogeneous amino-acid sequences. Instead, there were six positions in the α chain which could be occupied by more than one amino acid. The "interchangeable" amino acids in one rabbit were valine and leucine; leucine and phenylalanine; serine and threonine; valine and threonine; serine and leucine.

VON EHRENSTEIN offered the suggestion that the codons at these positions in the hemoglobin messenger paired with tRNAs for two different amino acids. This suggestion has two disadvantages:

(i) It does not explain why ambiguity should occur at only these positions when presumably there are other codons of identical composition that are not translated ambiguously.

(ii) The alternate amino acids at the "ambiguous" sites showed a pattern of genetic inheritance which corresponded to that expected from allelic genes.

It therefore seems logical to seek conventional genetic explanations for the results observed, i.e. that the ambiguity resulted from mutational base changes in the gene, and that one of the parents was a mutant, or that duplicate genes for the hemoglobin chains exist.

Support for both these hypothesis was offered by GARRICK and HUISMAN (1968) in studies with goat hemoglobin. Goats contain two types of hemoglobin, A and B. A single goat was found to have two different A hemoglobins, termed A and A′, the α chains of which differed in positions 19 (Glu and Ser), 26 (Ala and Thr), 113 (Leu and His) and 115 (Asn and Ser). The B hemoglobin of this goat contained an α chain which differed at position 75 from the αA chain (Asp and Tyr). A second goat was found to be homozygous for the αB chain and to have also the αA′ chain. This showed gene duplication for the α chain in the goat.

Other examples of similar phenomena are known in mice and in sheep. There is thus ample evidence for the existence of gene duplication as a continuing event in the hemoglobins, an addition to the well-known examples of it in evolution (INGRAM, 1963). Translational variation does not seem to be involved as a cause of differences between the hemoglobin chains.

In contrast, miscoding (also termed lack of fidelity) is a well-established phenomenon in the mechanisim of action of a number of the aminoglycoside antibiotics. The effect is exerted on the ribosome and as an example, streptomycin causes misreading in the messenger of U as C or A; and C as U or A. The effect of neomycin is even more marked and includes misreadings of C as G and U as G. "Distortion" of the ribosome appears to be the explanation.

The mechanism has been investigated by TRAUB et al. (1967). Streptomycin-resistant ribosomes, which are insusceptible to the inhibiting effect of streptomycin on protein synthesis, were dissociated into smaller units. The site of resistance was located in the 23 S component of the 30 S ribosomal subunit, and was subsequently identified as a protein, P_{10}, which permits various agents, including streptomycin, to induce translational ambiguity (OZAKI et al., 1969).

The findings show the 30 S ribosomal subunit participates in codon-anticodon recognition. Therefore codon-anticodon matching is not a simple pairing between a messenger RNA codon and the corresponding anticodon in tRNA, but a process which includes the influence of the ribosome.

Miscoding was observed in some of the earlier experiments on the code in which synthetic polynucleotides were used as templates. Poly-U was found

to code to a limited extent for leucine, if the magnesium content of the supernatant preparation was increased. Poly-U directs the formation of polyphenylalanine in a cell-free amino-acid incorporating system, and, by increasing the magnesium content of the system, some leucine was incorporated in addition to phenylalanine. Apparently some of the UUU codons were "read" as CUU. Evidently none of them were read as UCU, because no serine was incorporated (WEINSTEIN et al., 1966).

The effect of neomycin in the coding process is so drastic that it enables single-stranded DNA molecules to be "read" as messenger RNA (MCCARTHY and HOLLAND, 1965). BRETSCHER (1968) was able to use circular DNA, obtained from phage fd, as a template for polypeptide synthesis *in vitro* in the presence of neomycin.

Discussion and Summary

The current state of the code indicates that it is universal, and that codon-anticodon pairing follows the wobble rule. The chain-terminating codons are UAA, UAG and UGA, and the process of termination of polypeptides is carried out by two factors, R_1 and R_2, which are probably proteins. The initiation of polypeptide chains is associated with the codons AUG and GUG, which pair with formyl methionine tRNA to start such chains, although these codons pair with methionine tRNA and valine tRNA respectively in "internal" positions.

It does not seem possible at present to identify a sequence in tRNA which serves as a recognition site for the aminoacyl tRNA synthetase enzyme, and more knowledge of the tertiary structure of tRNA is needed.

The adenosine residue which characteristically adjoins the anticodon in tRNAs may be modified to conform with specific stereochemical requirements to stabilize "regular" base-pairing between the first base of the codon and the third base of the anticodon.

The evolution of tRNA is an unsolved problem which is closely related to the evolution of the code. The tRNAs for phenylalanine in wheat and yeast show homology which is similar to the case of the cytochromes *c* for these organisms, and the same homology exists in the case of serine tRNAs for yeast and the rat.

When such comparisons are made between yeast and *E. coli* tyrosine tRNAs, and between yeast and *E. coli* valine tRNAs, the homology is no greater than that between pairs of tRNAs for different amino acids picked at random. Therefore the evolutionary separation of the lines leading to bacteria and higher organisms may have taken place soon after the origin of tRNA, or alternatively the evolutionary differentiation of the tRNAs for *E. coli* and yeast, and for the tRNAs for different amino acids, may have reached a point of equilibrium.

The smaller (30 S) of the two ribosomal subunits participates in codon-anticodon recognition. A protein (P_{10}) which is concerned with this process has been identified.

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