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*With 44 Figures*

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# Subacute Sclerosing Panencephalitis: A Review[1]

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With 19 Figures

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## I. Introduction

Subacute sclerosing panencephalitis is an inflammatory, progressive, slowly evolving disorder of the central nervous system, affecting children and young adults. The disease has been known for almost forty years and described during this period under various designations by many neuropathologists. Each name, being based on different neuropathological findings, suggested a distinct nosological entity. Thus BODECHTEL and GUTTMANN (1931) described one case under the heading "diffuse encephalitis with sclerosing inflammation of the hemisphere white matter", DAWSON (1933, 1934) used the term "inclusion body encephalitis", PETTE and DÖRING (1939) named the disease "panencephalomyelitis" and VAN BOGAERT (1945) introduced the term "subacute

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sclerosing leukoencephalitis". Only during the last twenty years have various investigators, after careful studies, come to the conclusion that these conditions were actually the same disease—one that can result in a broad variety of neuropathologic changes. At the present time the descriptive term "subacute sclerosing panencephalitis", or SSPE, has been generally accepted as the designation for this disease.

The viral etiology of this disease was first suspected by Dawson (1933) because of his observation of intranuclear inclusion bodies in brain cells of patients with the disease. Pette and Döring (1939) also linked this disease with a viral agent, which they assumed to be the agent of Japanese B encephalitis. Several transmission experiments (see below) were attempted, but were substantially unsuccessful. Sherman et al. (1961) gave the search for a viral agent a more specific direction when they demonstrated, by the fluorescent antibody technique, herpes simplex antigen in the cytoplasmic inclusion bodies of nerve cells in the brain of a patient with SSPE. Other studies attempted to relate the disease to infectious hepatitis, coxsackie virus, attenuated poliomyelitis virus or postvaccinal reaction (Simpson, 1961; Toga and Martin, 1961; Caruso et al., 1964; Gulotta and Wechsler, 1964; Kolar et al., 1964).

A different concept of the etiology of the disease was based on the observation that an unusually high level of immunoglobulin G was present in the cerebrospinal fluid (CSF) of patients with SSPE. This almost universal finding was taken as an indication that this disease had allergy as its basis, the increase in the IgG being interpreted as an antibody response of the host to some antigen in the brain tissue (Pette, 1942).

None of these studies succeeded in isolating an infectious agent, in accomplishing transmission of the disease to an animal, or in demonstrating an immunologic disorder.

The first successful steps in the search for a viral agent were made by Bouteille et al. (1965) and Tellez-Nagel and Harter (1966b), who with an electron microscope, saw structures resembling nucleocapsids of a paramyxovirus in the inclusion bodies of brain cells of patients with SSPE. This observation was followed by the demonstration of relatively high measles antibody titers in the sera and CSF of these patients (Connolly et al., 1967; Freeman et al., 1967; Legg, 1967; ter Meulen et al., 1967) and of the presence of measles antigen in the brain (Connolly et al., 1967; Freeman et al., 1967). These pioneering studies formed the basis for an intensive analysis of the etiology and pathogenesis of this disease.

## II. Pathological Findings

A gross visual examination of brains of SSPE patients usually reveals marked atrophy of the cerebral cortex. The meninges appear brownish grey. Coronal section of the cerebral cortex usually reveals that the cortex is thin and that the white matter is dirty grey in color, and the ventricles are considerably enlarged. These observations are characteristic, although not patho-

gnomonic, and have been described by the earliest investigators of this disease (BODECHTEL and GUTTMANN, 1931; PETTE and DÖRING, 1939; VAN BOGAERT and DE BUSCHER, 1939; VAN BOGAERT, 1945). Moreover, they have been noted in most cases of SSPE that have been reported.

*Light microscopy.* Light microcropy reveals diffuse encephalitis of varying severity, which is seen in both the grey and white matter throughout the entire brain. [Thus the name, "panencephalitis", has been given to this disease; it was used for the first time by PETTE and DÖRING (1939), but was not generally accepted until recent years.] The cerebellum, however, is not affected in every case. It was completely free of lesions in one of the cases described by the authors (MÜLLER and TER MEULEN, 1969), whereas in several other cases reported (TARISKA, 1959; GUAZZI, 1961; JELLINGER and SEITELBERGER, 1967; SCHALTENBRAND et al., 1968) the encephalitic process was quite intense in the cerebellum and the lower brain stem. In addition to the cerebral cortex and white matter, which are the most severely involved, the basal ganglia, the thalamus, and the midbrain exhibit considerable pathological changes in nearly every case reported.

The encephalitic process in SSPE is characterized by perivascular cuffing, consisting of lymphocytes and plasma cells, and by a diffuse infiltration of the grey and white matter by these cells (Fig. 1a, b). In addition, a diffuse and nodular proliferation of glial cells (both micro- and macroglia), first described by BONHOFF (1948), is frequently observed (Fig. 1a). Perhaps the most striking feature of brain tissue of SSPE patients is the enormous increase in fibrous astrocytes, which form an extraordinarily dense network of fibers within the grey and white matter (Fig. 1c), especially within the centrum semiovale (Fig. 2a). The white matter shows slight demyelination of the sudanophilic type, which is not severe enough to explain the enormous fibrous gliosis (Fig. 2b). This discrepancy was noted earlier by VAN BOGAERT (1945), who for that reason introduced the name "subacute sclerosing leukoencephalitis" for the disease. Only a few cases with severe demyelination were reported (ALAJOUANINE et al., 1956; KRÜCKE, 1961; GULLOTTA and WECHSLER, 1964; GONATAS, 1966).

The nerve cells of the cerebral cortex are considerably diminished in number and in some very severe cases, even totally absent (BRAIN et al., 1948; TARISKA, 1959; MÜLLER and TER MEULEN, 1969). In the basal ganglia and within the brain stem, the neuronal loss is not as pronounced as in the cortex, but neuronophagia is frequently seen. Many of the ganglion cells show pathologic changes characterized by chromatolysis and hyperchromasia of the nuclei and an unusual homogeneity of the cytoplasm.

Another characteristic morphologic criterion for SSPE is the presence of intranuclear inclusion bodies of Cowdry type A (COWDRY, 1934), which were noted first by DAWSON (1933, 1934). These inclusion bodies have been found in nerve cells and astrocytes but are seen most frequently in the oligodendroglial cells. They are eosinophilic, when stained with hematoxylin and eosin, and

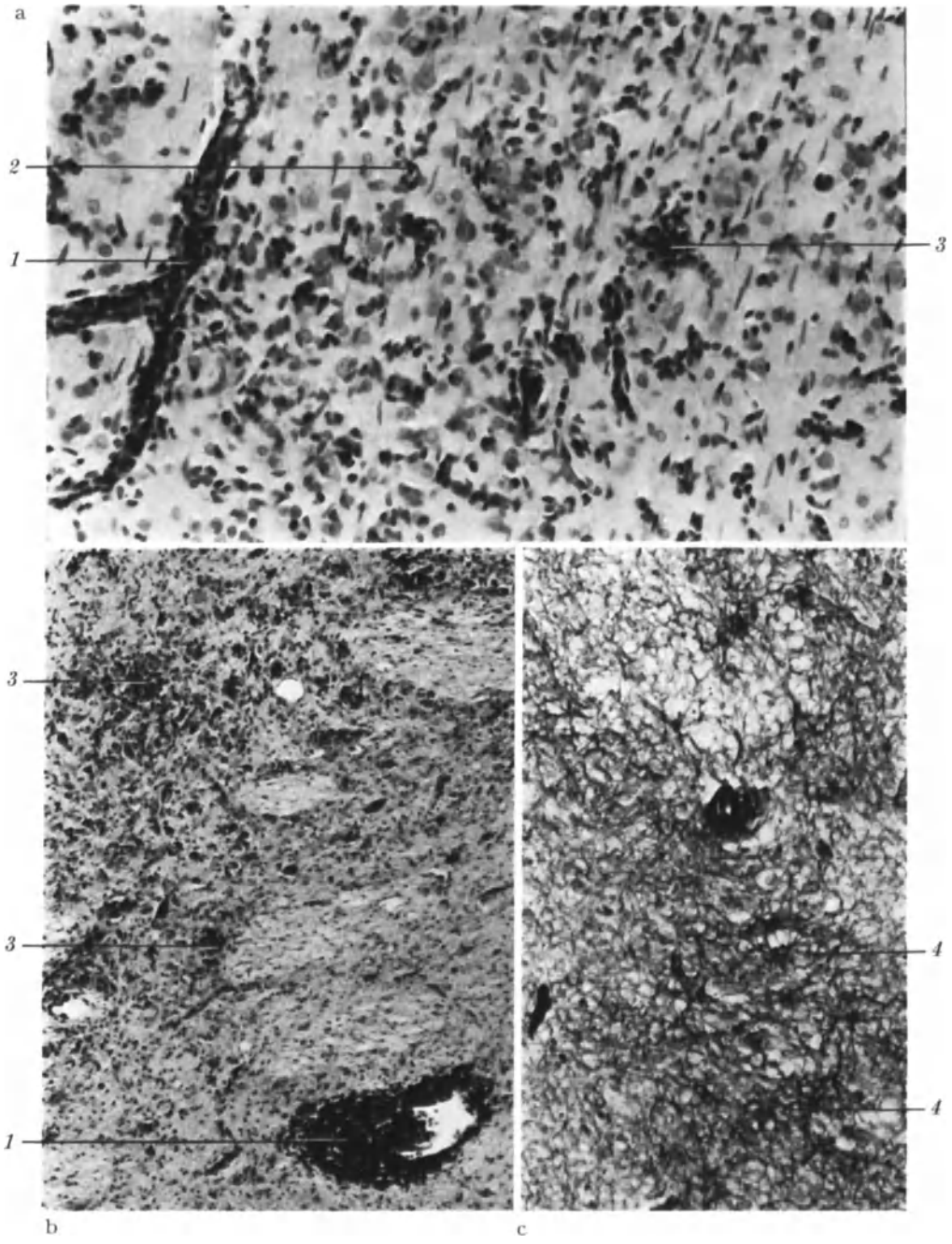
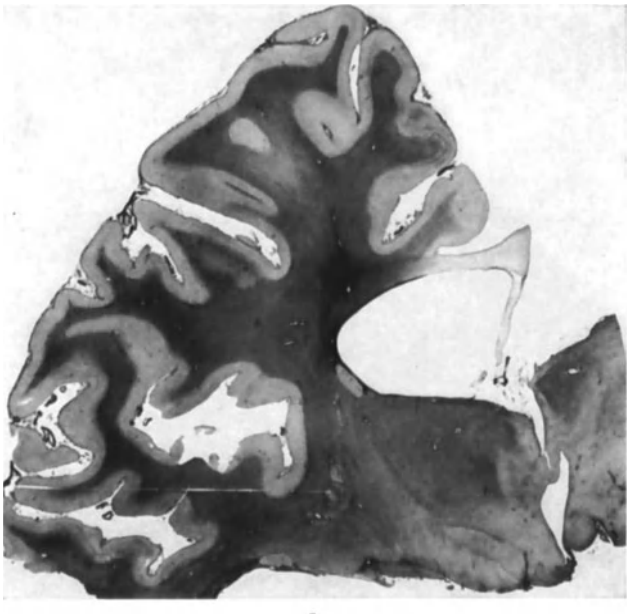
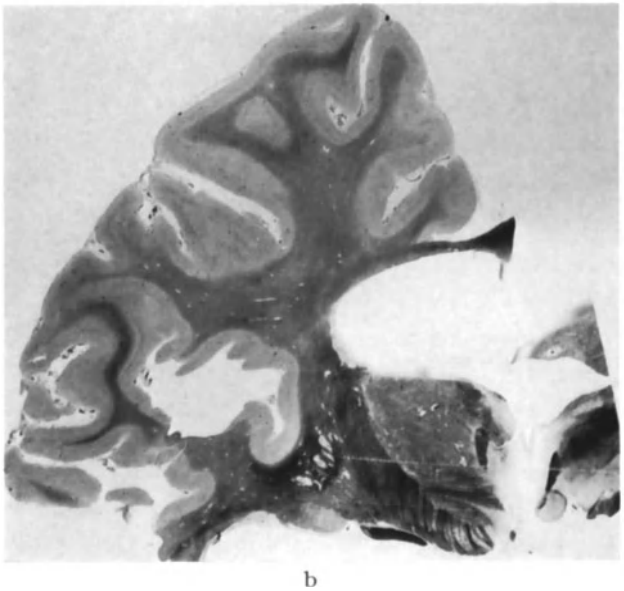



Fig. 1a–c. Human SSPE brain showing severe encephalitic process characterized by perivascular cuffing (*1*), diffuse infiltration by lymphocytes and plasma cells (*2*), diffuse and nodular proliferation of glial cells (*3*), and dense fiber gliosis (*4*). a) Temporal cortex, cresylviolet, ×225. b) Substantia nigra, cresylviolet, ×75. c) Lower midbrain, Holzer stain, ×225. (Reprinted with permission from Müller and ter Meulen, 1969)



a



b

Fig. 2a and b. Human SSPE brain. Coronal sections through the corpus mammillare showing an extraordinarily dense network of glial fibers within the centrum semiovale (a) and a slight demyelination of this area (b). a) Holzer stain, b) Myelin stain of Heidenhain-Woelcke. (Fig. 2a Reprinted with permission from MÜLLER and TER MEULEN, 1969)

occupy either the entire nucleus of the cell, pushing the chromatin aside toward the nuclear membrane (Fig. 3d), or else only a part of the nucleus, in which case they are themselves surrounded by a halo (Fig. 3a–c). The occurrence of inclusion bodies is quite variable. They have been noted as common in only a few instances (Dawson, 1934; Haymaker et al., 1958; Guazzi, 1961;

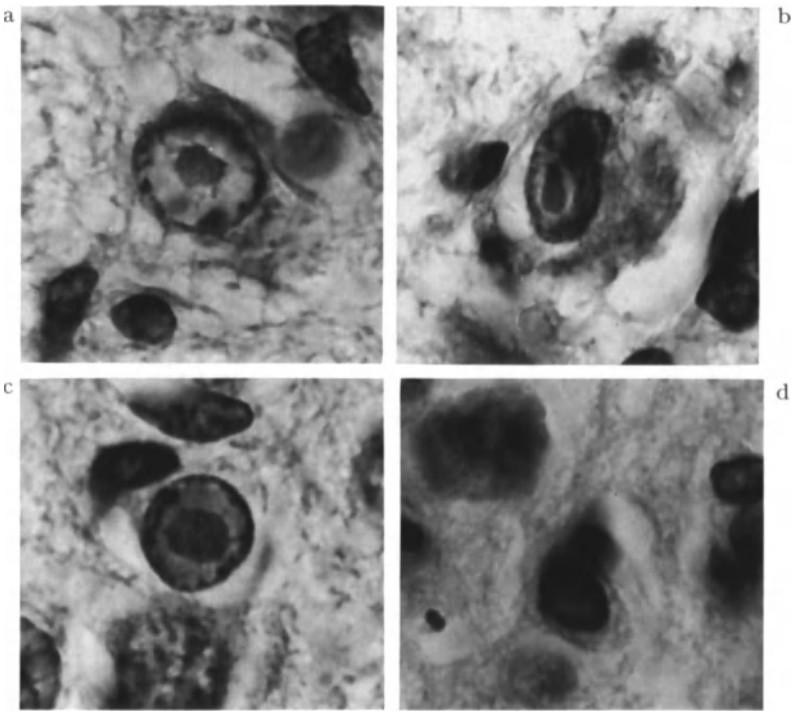



Fig. 3a–d. Typical eosinophilic intranuclear inclusion bodies within oligodendroglial cells in human SSPE. Hematoxylin and eosin (H.E.) stain, ×1800. a) Nucleoli form type (Cowdry type B). b) and c) Transitional stages of nucleoli form types to classical Cowdry type A inclusions. d) Late stage of type A inclusion which occupies almost the entire nucleus. (Modified micrograph from Müller and ter Meulen, 1969)

Seitelberger, 1961; Tellez-Nagel and Harter, 1966b; Ulrich and Kidd, 1966; Jellinger and Seitelberger, 1967). In some 50 to 60 percent of the reported cases, they were scanty and seen only in the oligodendroglial cells; in approximately 30 percent they were not noted at all. van Bogaert (1958, 1960) reported that in a reinvestigation of 21 of his original cases in which he had not noted any inclusion bodies, only five cases did have typical inclusion bodies. Several authors (Brain et al., 1948; Greenfield, 1963; Jellinger and Seitelberger, 1967; Herndon and Rubinstein, 1968) have reported cytoplasmic inclusion bodies predominantly within nerve cells and on the basis of this finding have made a strong argument against the hypothesis that herpes simplex virus is a possible agent of SSPE, because this virus never produces

cytoplasmic inclusion bodies. The seemingly preferential involvement of oligodendroglial cells has suggested to some authors (HAYMAKER et al., 1958; SEITELBERGER, 1961; JELLINGER and SEITELBERGER, 1967) that this mechanism is responsible for demyelination in SSPE. In addition, the demyelination must also result in a secondary manner from neuronal loss and thus might be

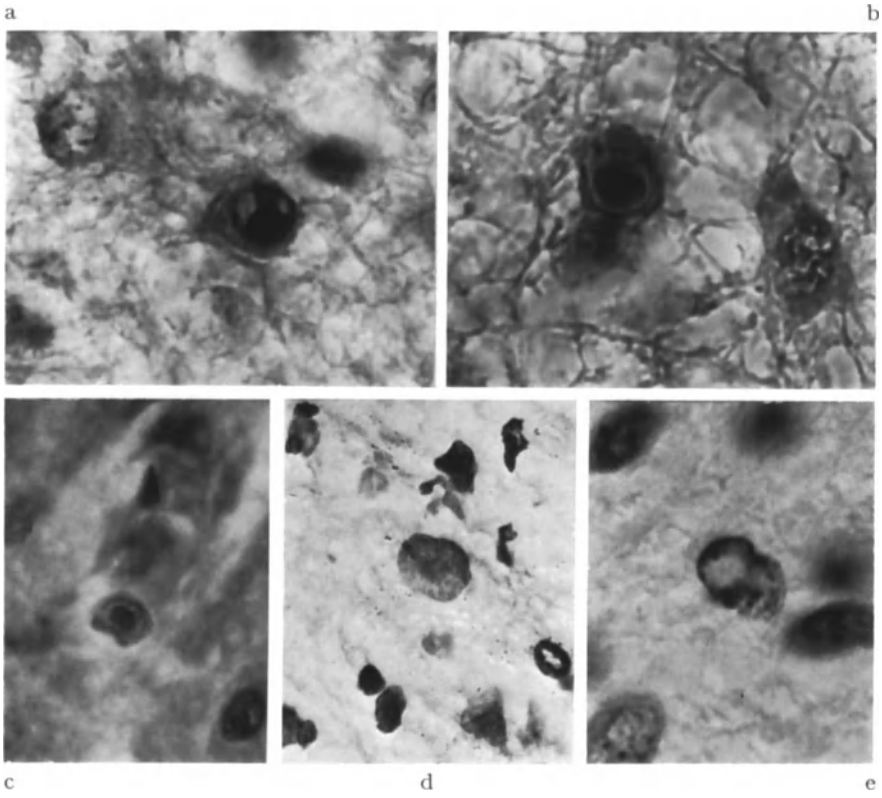



Fig. 4a–e. Histochemistry of intranuclear SSPE inclusion bodies, showing positive staining reactions with a) Periodic acid Schiff (PAS) for mucopolysaccharides, ×1250, b) Ninhydrin Schiff for amino acids, ×1650, c) Pyronine in the methyl green-pyronine staining, showing the RNA content, ×1000, and d) Toluidine blue and ammonium molybdate, method C for ribonucleoproteins, ×1000. e) Negative staining in the Feulgen reaction, ×1500. (Modified micrograph from MÜLLER and TER MEULEN, 1969)

considered a type of Wallerian degeneration within the cerebral white matter (GONATAS, 1966). White matter edema may be a third factor which leads to a breakdown of myelin sheaths in SSPE (KRÜCKE, 1957).

*Histochemistry.* Histochemical investigations in SSPE have been restricted mainly to the study of the inclusion bodies (SHIRAKI and YAMAMOTO, 1962; BOZSIK et al., 1963; HERNDON and RUBINSTEIN, 1968). The typical Cowdry type A inclusion bodies have been stained positively with periodic acid Schiff (PAS) (Fig. 4a), ninhydrin-Schiff (Fig. 4b), the coupled tetrazonium reaction, the Millon reaction, methylene blue following acetylation according to the

method of Deitch (1964), pyronine in the methyl-green pyronine staining (Fig. 4c), toluidine blue and ammonium molybdate (Fig. 4d) according to the method of Love and Walsh (1963), and other staining techniques which have been used to reveal ribonucleoprotein (RNP) and proteins. These inclusion bodies failed to take up methylene blue, after the ribonucleic acid (RNA) had been removed with RNase or HCl (Deitch, 1964), and were not stained with Feulgen reaction (Fig. 4e) or the staining methods used to detect lipids, such as Sudan III, oil red O, Sudan black B, Nile blue sulphate or acetic cresyl violet (Müller and ter Meulen, 1969). Under polarized light microscopy they did not show birefringence. A number of authors have concluded therefore that the intranuclear inclusion bodies in SSPE consist of carbohydrates, RNA, RNP, and proteins, and do not contain deoxyribonucleic acid (DNA) or lipids (Shiraki and Yamamoto, 1962; Bozsik et al., 1963; Herndon and Rubinstein, 1968; Müller and ter Meulen, 1969). Several observers (Stammler and Fotakis, 1964; Spaar, 1965; Jellinger and Seitelberger, 1967; Zu Rhein and Chou, 1968) have criticized the concept of the viral origin of the inclusion bodies, as noted by Dawson (1933, 1934) and others (Malamud et al., 1950; Weingarten and Seitelberger, 1952; Haymaker et al., 1958; Krücke, 1960) and have considered these bodies to be the result of a severe, but nonspecific disturbance of the cellular metabolism of proteins. On the basis of recent ultrastructural and immunochemical studies, others (Bouteille et al., 1965; Tellez-Nagel and Harter, 1966b; Dayan et al., 1967; Herndon and Rubinstein, 1968; Müller and ter Meulen, 1969; Toga et al., 1969) have considered the synthesis of these inclusion bodies a direct consequence of the infection of the cell with a virus, very likely a RNA measles-like virus. This is consistent with the high RNA content of the intranuclear inclusion bodies (ter Meulen et al., 1970c) and the fact that most of the protein consists of histone-free proteins (Sandritter et al., 1960).

*Quantitative Cytochemistry.* When nerve cells in brains of SSPE patients were analyzed cytochemically in our laboratories (Müller and ter Meulen, 1969; ter Meulen et al., 1970c), the cytoplasm of the infected ganglion cells stained more intensely than that of comparable cells in non-SSPE brain tissues. This held true when tests were made for amino acids, proteins, RNA and RNP. Staining for lipids and lipochromes showed no increase of these substances in the affected cells; moreover, no birefringence was noted. These changes were also quantitated by cytophotometry with a scanning microspectrophotometer (UMSP I, Zeiss and Co.) and thus a new approach to the cytochemical analysis of brain tissue became possible. Our investigations carried out on isolated and unstained, or specifically stained, nerve cells, using ultraviolet and visible light spectra (Neuhoff et al., 1968), showed in the SSPE cells an increase in proteins of 40% over the control cells, an increase in RNA of 34%, and a decrease in DNA of 29% (ter Meulen et al., 1970c) (Fig. 5).

The data recorded by the scanning cytophotometer were formulated into histograms by the computer. This method permitted identification of various cell components in SSPE through the pattern-recognition technique (Wied

et al., 1968). In this way infected cells could be compared with control cells and the distribution of intracellular abnormalities could be ascertained. The computer analysis revealed marked changes of the SSPE nerve cell nuclei, which could not be detected under an ordinary light microscope. These changes were shown to be the beginning of the formation of the intranuclear inclusion bodies. It appeared, therefore, that the increase in the RNA was due to the presence of viral nucleic acids, and the increase in the protein content was due to the presence of virus-specific antibodies.

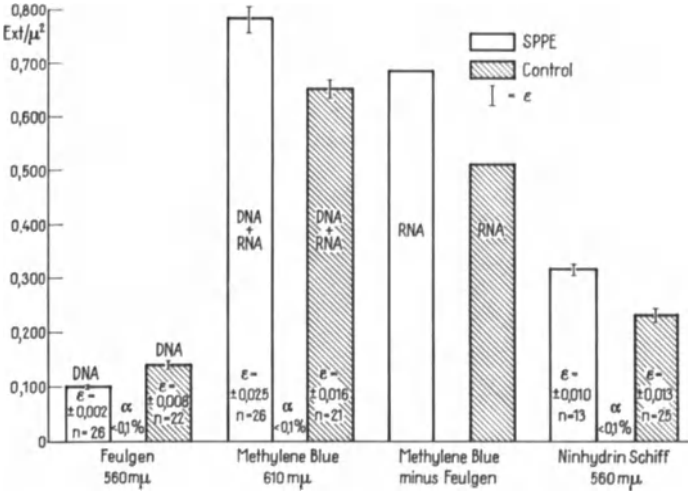



Fig. 5. Quantitative cytochemistry of SSPE nerve cells. Mean extinction values for field of measurement of isolated stained nerve cells showing the increase of RNA and proteins in SSPE nerve cells in comparison with control cells. (Reprinted with permission from TER MEULEN et al., 1970c)

*Electron microscopy*. The initial investigation of SSPE by electron microscopy was by BOUTEILLE et al. (1965). These authors described the microtubules within the inclusion bodies and suggested that they resembled nucleocapsids of a paramyxovirus. This observation was later confirmed by TELLEZ-NAGEL and HARTER (1966a, b); by PERIER et al. (1967) and by SHAW et al. (1967). Other investigators described "virus-like" particles that did not resemble paramyxovirus structures, but had some morphologic similarities to herpes simplex or herpes-B virus (GONATAS and SHY, 1965; GONATAS, 1966; GONATAS et al., 1967; ULRICH and KIDD, 1966). ZU RHEIN and CHOU (1968) did not detect any viral structures in a SSPE biopsy specimen they investigated. They did note some aberrations in the ultrastructure of the nerve and glial cell nuclei, but interpreted them to be nonspecific changes, perhaps related to a viral infection.

In a detailed study HERNDON and RUBINSTEIN (1968) demonstrated that the viral particles they observed in biopsy specimens of three patients with SSPE were indistinguishable from large myxovirus particles. They described

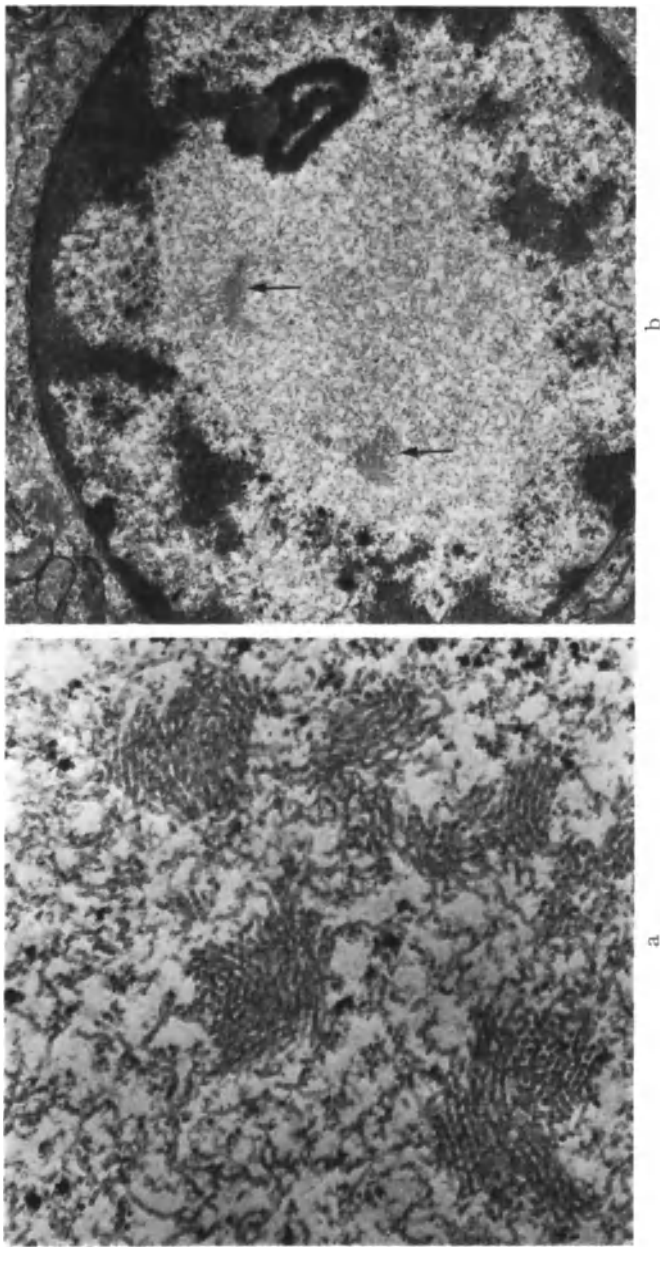



Fig. 6. Nuclear changes of oligodendrocytes in the grey (a) and white (b) matter of a human SSPE brain. a Nucleocapsids occupy the central part of the nucleus. They are randomly arranged with array formations (arrows). ×13800. b Arrays of tubules in the nucleus of another oligodendrocyte. Outer diameter, 170 Å; inner diameter, 70 Å. ×90000. (Reprinted with permission from Katz et al., 1969b)

a sequence that began with intranuclear inclusion bodies in the shape of nucleoli and ended in multitubular ones. The authors hypothesized that these tubules disrupt the nuclear membrane and extend, as part of their further development, into the cytoplasm. The nucleoli-form inclusion bodies were correlated by them with the Cowdry type B inclusion bodies and were found to contain a high concentration of RNA. In contrast, the multitubular in-

clusion bodies were related to the Cowdry type A inclusion bodies and were found to contain only a small concentration of RNA. The cytoplasmic inclusion bodies were rich in RNA and proteins. In this electron microscope study the nucleoli-type inclusion bodies were found to consist of dense granules 300–500 Å in diameter, either densely packed or loosely scattered within the inclusion. The inclusions were surrounded by a clear halo composed of filamentous material of 20–40 Å in diameter. The multitubular type of inclusion body consisted of tubules of various lengths, having an inside diameter of 40–60 Å and an outside diameter of 170–190 Å. The tubules were occasionally closely packed in a pseudo-crystalline array, but more often they were randomly scattered within the inclusion bodies (Fig. 6). The inclusion bodies themselves were as large as 12 μ and often filled almost the entire nucleus.

The cytoplasmic inclusion bodies which appeared frequently in oligodendroglial and nerve cells that contained the multitubular type were composed of a dense filamentous material which at a higher magnification was seen to consist of the 170–190 Å tubules described in the intranuclear inclusion bodies. However, granular material was densely superimposed on them.

In addition, HERNDON and RUBINSTEIN (1968) observed particles of 1400 to 2500 Å in diameter budding from the cytoplasmic inclusion bodies into the dilated spaces of the endoplasmic reticulum. The particles were surrounded by membranes which were indistinguishable from the plasma membrane and were composed of the same tubules as seen in the cytoplasmic inclusion bodies.

Recently these findings were confirmed by TOGA et al. (1969) and OYANAGI et al. (1971).

## III. Serology

In most patients with SSPE, high titers of antibodies against measles virus were found in the serum and spinal fluid at some time during the disease. Such high levels of antibodies in the serum have not been seen during or following the course of natural measles (Fig. 7) or after vaccination with live attenuated or killed (TER MEULEN et al., 1968b) measles virus. Antibodies against measles have been seen in the cerebrospinal fluid only in SSPE and in two, reported, exceptional cases of multiple sclerosis (GIBBS et al., 1969) where their level was very low. These high antibody titers in cases of SSPE have been reported by many investigators (CONNOLLY et al., 1967; DAYAN et al., 1967; FREEMAN et al., 1967; LEGG, 1967; TER MEULEN et al., 1967, 1968b, 1969; ADELS et al., 1968; BERMAN et al., 1968; GRIFFITH and KATZ, 1968; LENNETTE et al., 1968; SEVER and ZEMAN, 1968) who found them by every method that was used for their detection, i.e., complement fixation (CF), hemagglutination-inhibition (HAI), neutralization (N), and fluorescent microscopy. In general, the fluorescent antibody (FA) test gave the highest titers and has seemed to be the most sensitive assay. In some studies, serial serum specimens taken during the course of the disease showed significant increases in complement-fixing and hemagglutination-inhibiting antibody levels; on the other hand, the FA titers remained relatively fixed (LENNETTE et al., 1968; CONNOLLY et al., 1967). In

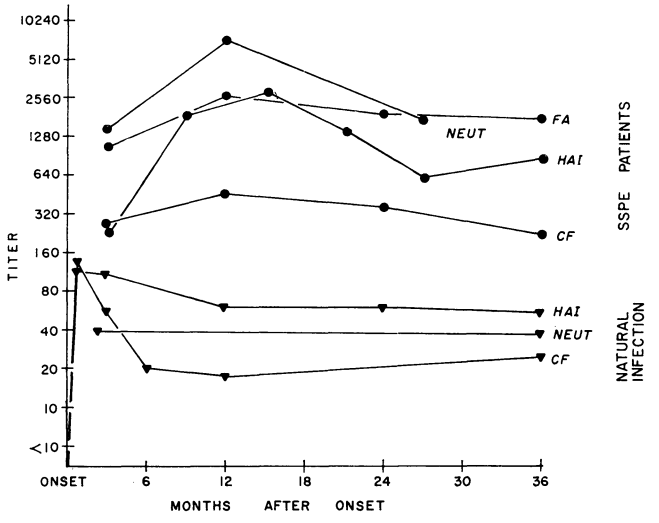



Fig. 7. Comparison of the geometric mean values of *HAI, CF, FA* and *N* antibody titers to measles virus in the sera of patients with SSPE to titers in the sera of humans following natural acute measles infections. (Reprinted with permission from ADELS et al., 1968)

other studies (ADELS et al., 1968; GRIFFITH and KATZ, 1968; SEVER and ZEMAN, 1968; TER MEULEN et al., 1968a, 1969), however, specimens taken at different intervals revealed measles antibody titers to be essentially unchanged in all four test procedures. To exclude other myxoviruses as possible causative agents in this disease, serological tests for influenza A and B, parainfluenza 1, 2, 3, 4 and SV5, and mumps virus were performed (ADELS et al., 1968; LENNETTE et al., 1968; SEVER and ZEMAN, 1968; TER MEULEN et al., 1969). In addition, tests were made for antibodies to herpes simplex virus, varicella virus (ADELS et al., 1968; LENNETTE et al., 1968; SEVER and ZEMAN, 1968), rubella (ADELS et al., 1968; TER MEULEN et al., 1969), respiratory syncytial virus (ADELS et al., 1968; TER MEULEN et al., 1969), adenovirus, ECHO virus 6, coxsackie A, B (SEVER and ZEMAN, 1968) and certain arboviruses (ADELS et al., 1968). The results showed that antibodies to these viruses were not present consistently and the titers demonstrated were relatively low.

Although the source of measles antibodies in the CSF of SSPE patients has not been determined definitively, they most probably derive from the brain tissue and are not merely a reflection of the high levels of antibodies in the serum (CUTLER et al., 1968; TOURTELLOTTE et al., 1968; TER MEULEN et al., 1968b, 1969). The ratio between CSF and serum antibodies is of the order 1/50 and much higher than would be expected on the basis of the CSF-to-serum ratio in, for example, poliomyelitis (CLARKE et al., 1965).

The cross-reaction of measles antibodies and canine distemper virus in SSPE sera was investigated by LENNETTE et al. (1968), who used the indirect fluorescent antibody method in parallel with the fluorescent antibody test for measles. These authors showed that the levels of antibody to distemper virus were essentially the same as the measles antibody titers. In the studies of

BECH (1960) and BLACK and ROSEN (1962) of human sera obtained from the populations of Greenland and Tahiti, respectively, where no known exposure to distemper virus has occurred, the CF and neutralizing antibody (NA) tests revealed lower titers of distemper antibody than measles antibody. No comparison between the measles CF and NA titers in SSPE sera and those of distemper virus antibodies has thus far been made, but it seems most likely that the distemper antibody titers demonstrated in the SSPE sera by the FA test can be accounted for by the measles antibody titers, since the immunologic relationship between these two viruses is well established. Antibodies to distemper are common in man, in spite of the fact that distemper is not known to infect man, and their presence can always be correlated with the presence of measles antibodies.

The standard assay procedure, using wild or attenuated measles virus as antigen, did not reveal any qualitative differences between SSPE sera and sera of subjects who had natural measles or had been immunized against measles. The first indication of some differences between these sera was noted when the indirect immunofluorescence tests were applied to tissue cultures derived from SSPE brain specimens. These tissue cultures appeared to be persistently infected with an agent that caused formation of giant cells, spindle cells, syncytia and many intranuclear and cytoplasmic inclusion bodies. The giant cells contained a measles-like antigen detectable by measles antiserum. In the indirect immunofluorescence test, depending on the serum used, two types of fluorescence were noted: cytoplasmic and nuclear (Fig. 8) (TER MEULEN et al., 1970a). The cytoplasmic fluorescence showed antigens aggregated in large globules throughout the entire cytoplasm. The nuclear fluorescence appeared either in a speckled form or in the shape of an inclusion body. Sera of SSPE patients, regardless of the time of collection during the course of the disease, always produced cytoplasmic *and* nuclear fluorescence. The same result was noted with sera from patients who had recently had measles (up to four months before the test), but the titers were lower than those in the sera from SSPE patients. In sera obtained from subjects five months or more after clinical measles, as well as sera of rabbits immunized against split antigen of measles, only cytoplasmic fluorescence was seen. Thus a qualitative difference exists between the sera from SSPE patients and the other sera, in that the latter elicited no nuclear fluorescence in the test described (KATZ et al., 1970a). The failure of sera from patients in the late convalescent stages of measles to elicit fluorescence cannot be accounted for merely by the relatively lower titers of antibodies, because nuclear fluorescence is demonstrable in the sera of patients with measles within even a few days after the onset of the disease, when cytoplasmic fluorescence titers are quite low. Moreover, the CSF of patients with SSPE also contained antibodies reacting with the nuclear antigen, even though all categories of measles antibodies were much lower than those in the serum.

Although the mechanism which produces nuclear fluorescence is unknown, it appears likely that the antigen responsible for it is related to an early viral

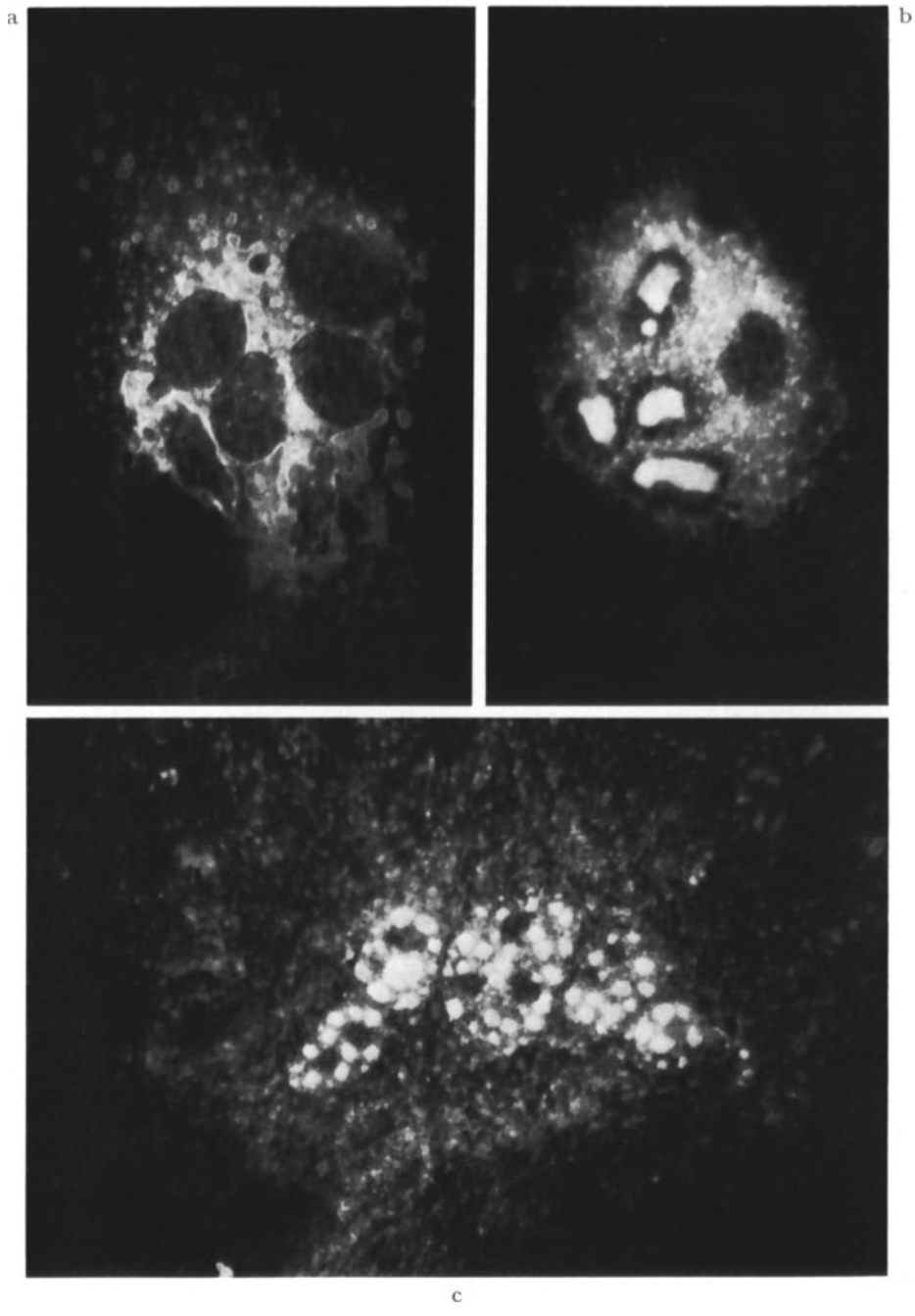



Fig. 8a–c. Giant cells from tissue cultures derived from SSPE brain biopsies. a) Cytoplasmic fluorescence after staining in the indirect immunofluorescent test with measles serum from patients more than 5 months after measles. ×750. b) Cytoplasmic and inclusion type of intranuclear fluorescence after staining in the indirect immunofluorescence test with SSPE serum. ×600. c) Cytoplasmic and speckled intranuclear type of fluorescence after staining in the indirect immunofluorescent test with SSPE serum, ×750. (Fig. 8b reprinted with permission from Oyanagi et al., 1970)

component and that the antibody against it wanes when the stimulus of active viral replication has ceased. An alternative explanation is that the infectious virus induces an antigen that gives rise to the antibody against the intranuclear material and that this antigen disappears in the course of normal measles infection, but persists during SSPE.

## IV. Immunofluorescence of Brain

The immunofluorescent technique was very useful in demonstrating measles antigen in brain sections of SSPE tissues, whereas other serologic methods failed (CONNOLLY et al., 1967; FREEMAN et al., 1967; LENNETTE et al., 1968; TER MEULEN et al., 1969). The treatment of brain homogenates with trypsin, Tween ether or alkaline solutions did not yield a high titer of hemagglutinating (HA) or CF measles antigen (TER MEULEN et al., 1969). These failures may be explained by the presence of a high level of measles antibodies in the brain extract. The antibody levels in extracts of different brain regions did not differ in any of the four serologic tests from those of the spinal fluids, and these antibodies probably masked the CF and HA measles antigen present in the brain homogenates. Measles antigen in SSPE ganglion and glial cells was detected by the indirect and direct immunofluorescent techniques, by using monkey, guinea pig, and human measles-immune sera. Particles characterized by brilliant staining and masses of homogeneously stained antigen could be detected in the cytoplasm and the nuclei of nerve and glial cells, as well as a number of cells that could not be identified under the fluorescent microscope (Fig. 9). The specific staining sometimes extended into the nerve cell processes. Often the cytoplasmic staining assumed the shape of inclusion bodies. The number of cells showing fluorescent staining and the amount of antigen in the cell differed from specimen to specimen. The extent of the involvement of brain areas visualized by fluorescent microscopy, appears to be correlated with the frequency and size of the inclusion bodies seen within those areas by either electron or light microscopy. Areas with many inclusion bodies showed intense staining in many cells, whereas areas with few or no inclusion bodies had virtually no immunofluorescence. It is noteworthy that inclusion bodies detected in abundance within an area of the brain at the time of the biopsy often had vanished from the same area of the brain when it was examined after autopsy.

It is of interest that human anti-measles sera produce more brilliant staining in the indirect immunofluorescent test than the sera against measles prepared in animals. This suggests that measles antigen located in the nerve cells is partly blocked by the patient's own measles antibodies (LENNETTE et al., 1968; TER MEULEN et al., 1969). When serum with antibodies against herpes simplex, varicella, or distemper virus was applied, no specific fluorescence resulted (LENNETTE et al., 1968). The latter can be explained by the finding of GILLESPIE and KARZON (1960) and ROBERTS (1965) that measles infection

induces antibodies that cross-react with distemper antigen, whereas infection with distemper virus induces only homotypic antibodies.

The presence of immunoglobulins in brains of SSPE patients was demonstrated by several methods. Biochemical studies showed an increase in proteins and related it to the gamma-globulin (Tourtellotte et al., 1968). Immunofluorescent assays revealed IgG in plasma cells and lymphocytes of brain sections from autopsy material (ter Meulen et al., 1967; ter Meulen et al.,

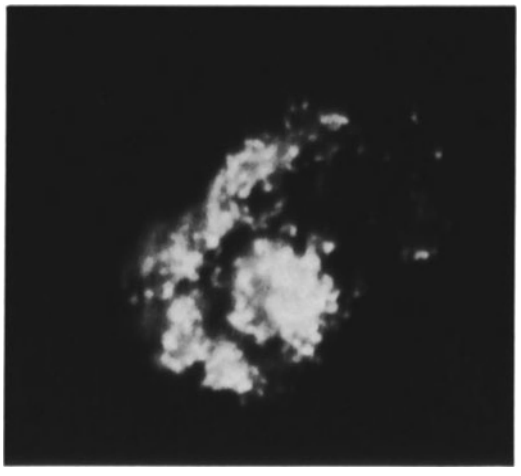


Fig. 9. Intracellular fluorescence in the cytoplasm and nucleus of nerve cell after staining in the indirect immunofluorescent test with human measles immune serum, taken 10 weeks after the onset of clinical measles ("early convalescent serum"). SSPE brain cryostate section. Vertical ultraviolet illumination, ×1600

1968a, 1969; Vandvik, 1970). The areas of perivascular cuffing and similar infiltrations of the leptomeninges showed preferential staining (Fig. 10). In addition, many nerve and glial cells in the brain cortex and midbrain showed specific staining by anti-human-IgG and anti-beta-1C-globulin, characterized by granular fluorescence in the cytoplasm and occasionally also in the nucleus (ter Meulen et al., 1967, 1968a, b, 1969; Vandvik, 1970). The staining also extended into the cell processes (Fig. 11). The presence of IgG and beta-1C-globulin points to the presence of antigen-antibody complexes in the cytoplasm or nucleus of nerve and glial cells. The dissociation of these complexes by treatment of unfixed frozen brain sections with high molecular salt solutions of thiocyanate or perchlorate, according to the method of Dandliker et al. (1967) and Edgington et al. (1967), showed the antigen to be a measles antigen (ter Meulen et al., 1969). Sections treated so as to remove the IgG, and stained with a fluorescein-labeled serum against measles, gave staining of equivocal specificity (ter Meulen et al., 1969) (Fig. 11b).

Whether these measles antibody complexes are formed in the presence of complement while the patient is alive, or only post mortem, could not be clearly established because IgG and complement could be demonstrated in

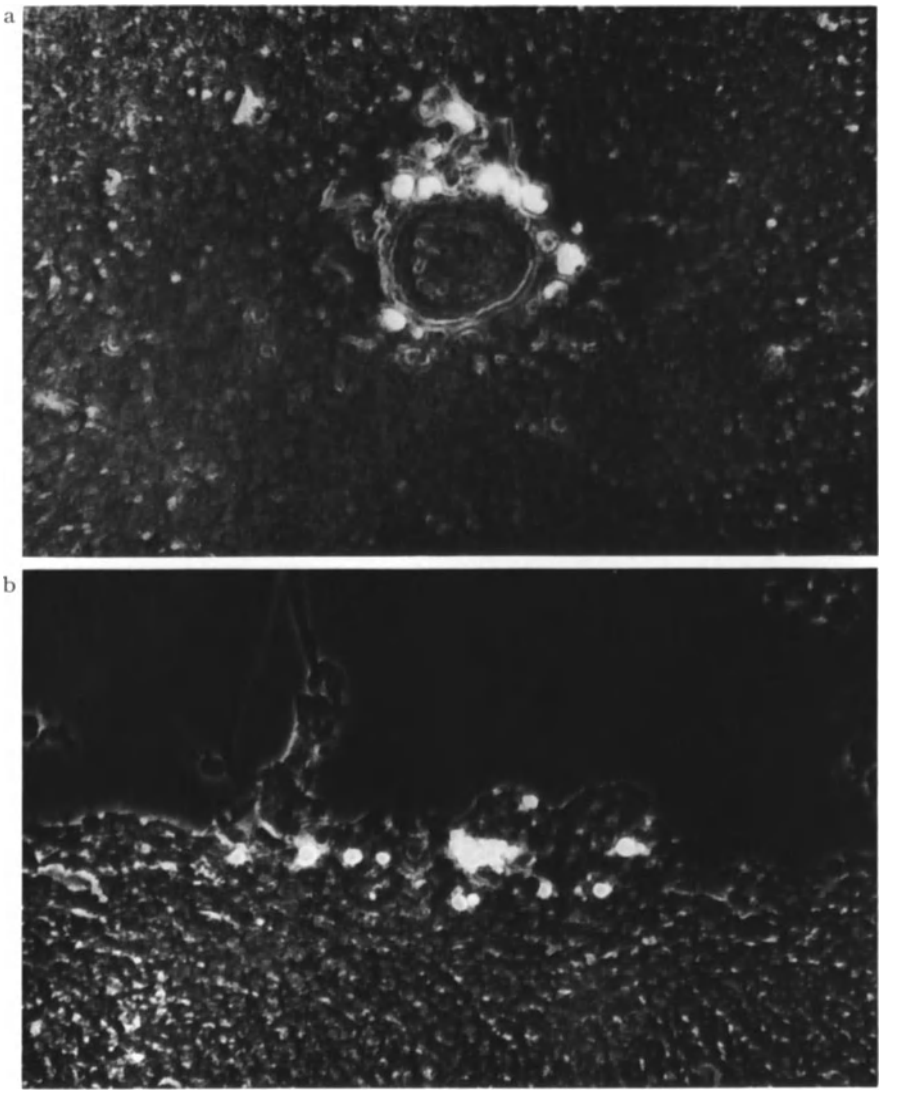
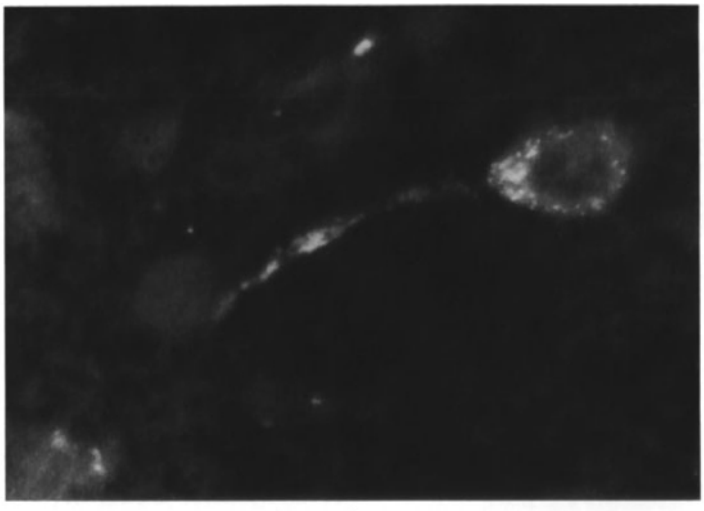


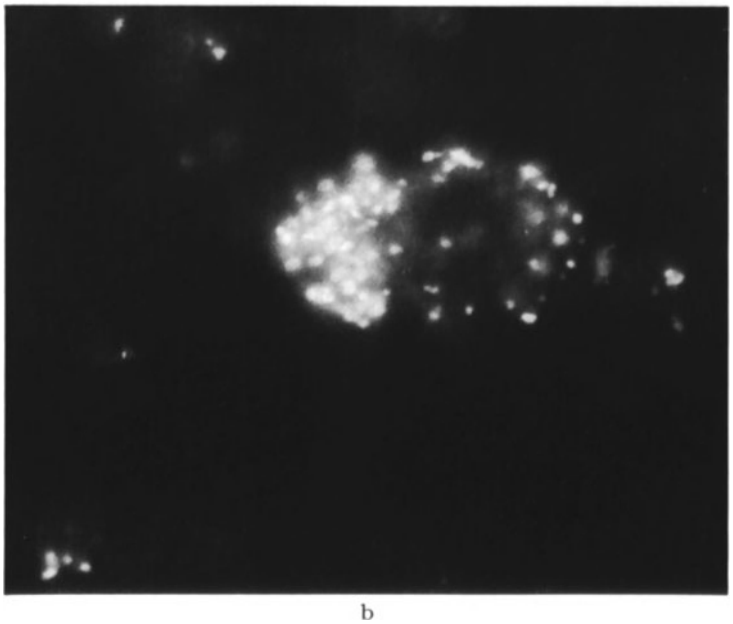

Fig. 10a and b. Fluorescent lymphocytes and plasma cells. Staining with conjugated rabbit anti-human gamma-globulin. Carnoy-chloroform fixation. Phase contrast combined with vertical ultraviolet illumination, ×360. a) Perivascular cell infiltration of the brain stem. b) Cellular infiltration of the leptomeninges. (Reprinted with permission from TER MEULEN et al., 1969)

only a few biopsies. However, if present during the disease process, these immunologic complexes could well destroy cell membranes and be responsible for the destruction of nerve and glial cells seen in the neuropathological examination of this disease.

The lymphocytes and plasma cells, forming inflammatory infiltrates in SSPE brains and shown to be rich in IgG, may be the source of the immuno-



a



b

Fig. 11. a) Intracellular fluorescence in the cytoplasm, perinuclear region, and dendrites of a nerve cell of the brainstem stained with conjugated rabbit anti-human gamma-G globulin. Sections of freeze dried tissue. Vertical ultraviolet illumination, ×1230. b) Nerve cell in the nucleus of the occulomotor nerve with granular fluorescence of the cytoplasm. Cryostate section. Staining with conjugated rabbit anti-measles serum after treatment of the section with chaotropic ions. Vertical ultraviolet illumination, ×1600. (Reprinted with permission from TER MEULEN et al., 1969)

globulins found in brain extracts and the CSF of the patients. CUTLER concluded from his studies of radioactive IgG infusion in patients with SSPE that the brain IgG was synthesized locally (CUTLER et al., 1968). TOURTEL-

LOTTE et al. (1968) calculated from protein determination in the blood and brain tissue of patients with SSPE that approximately 95 % of the IgG in the brain was produced in the central nervous system.

## V. Search for the Virus

Once the concept of viral etiology was seriously established, it remained only for a patient and intensive search to lead to the discovery of an infectious agent.

*Animal inoculation.* The initial attempt to transmit an infectious agent, in the infancy of virology, was inevitably limited to animal inoculation. These studies were undertaken by DAWSON (1933) as the result of his original observation of viral imprints on the brain tissue, the inclusion bodies. He inoculated hamsters, mice, and guinea pigs but observed no evidence of disease or any histopathological changes that might suggest a sub-clinical infection. He did not attempt serial blind passages. The next recorded attempt was by MARTIN et al. (1950) who serially inoculated mice, but again produced neither a clinical disease nor histopathological changes. Some years later PELC et al. (1958) again made an attempt at transmission of SSPE to animals and their effort brought some, albeit fleeting, success. Rhesus monkeys that received intracerebral inoculations of a brain homogenate from a patient with SSPE developed microscopic lesions characterized by rare inclusion bodies in the astrocytes. Curiously enough, the animals that were sacrificed on the eighteenth day post inoculation had remained well. An attempt at passage of an agent from these brains by inoculating homogenates into mice and hamsters failed. The authors were unable to continue the inoculations into monkeys and therefore terminated this experiment. They were also unsuccessful in the attempt to isolate an infectious agent *in vitro*. As a consequence of the revival of interest in SSPE five years ago, ADELS and his colleagues (1968) embarked on a veritable hunt for a transmissible agent, but were unsuccessful, reporting failure to transmit an agent to a large array of animals, ranging from baboons through goats and sheep and pigs to domestic fowl. In 1968 KATZ et al., in a preliminary report, described transmission of a slowly developing encephalitis in ferrets inoculated intracerebrally with homogenates of human SSPE brain tissues obtained at a diagnostic biopsy. The incubation period was about five months, but especially noteworthy was the fact that the animals did not have obvious clinical symptoms and were recognized as being somewhat unwell only by a certain apathy that characterized their approach to food and their response to noxious stimuli. Electroencephalographic (EEG) tracing of ferrets anesthetized with barbiturate revealed that the animals inoculated with SSPE brain homogenates, but not the controls (both uninoculated and inoculated with non-SSPE brain material), showed EEG changes (Fig. 12). The brains of ferrets sacrificed at this point in their disease showed only minimal inflammatory changes, but had pronounced gliosis (Fig. 13). With the serial passage of ferret brain homogenates into other ferrets, the disease was reproduced after a shorter

Table 1. *Inocula that produced encephalitis in ferrets.*
(Reprinted with permission from Katz et al., 1970)

| Inoculum | Approximate incubation period (weeks) |
|---|---|
| SSPE human brain homogenate | 20 |
| SSPE ferret brain homogenate | 12 |
| SSPE human brain cell culture | 2 |
| SSPE virus in AGMK cell culture | 2 |

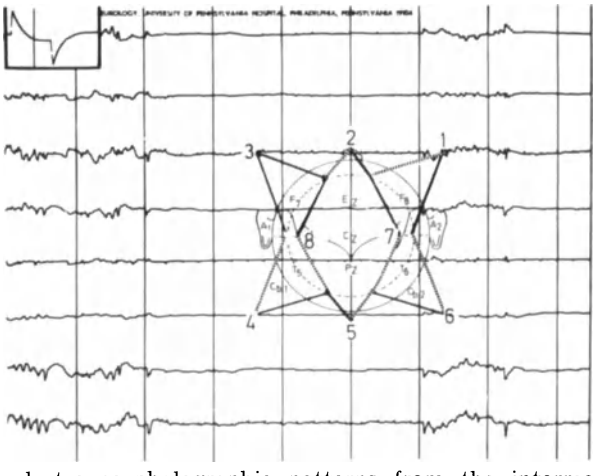


Fig. 12. Typical electroencephalographic patterns from the intermediate phase of a ferret inoculated with human SSPE brain. Well-developed burst suppression pattern and characteristic bifrontal sharp and slow waves are apparent. (Reprinted with permission from Katz et al., 1968)

incubation period (Table 1) and a more intense response occurred in the brain tissue, which was now characterized by considerable inflammation and the presence of eosinophilic intranuclear inclusion bodies (Fig. 14). The clinical features of the disease, however, remained quite subtle. Animals with the encephalitis that were not killed, did not die, but ultimately regained their interest in food and became more alert. The brains of several such animals, examined a year or more after inoculation, showed some atrophy of the brain tissue, residual gliosis, and a considerable loss of neurons (L. B. Rorke and M. Katz, unpublished data). None of this series of ferrets developed antibodies against measles, but it must be pointed out that ferrets have a poor serologic response to measles. All attempts at isolation of the infectious virus by the inoculation of ferret brain homogenates into a variety of tissue cultures met with failure. Attempts at transmission of the encephalitis into suckling and adult hamsters, rats and mice also failed, even after serial blind passage in these animals.

Following development of brain explant cell cultures and the isolation of a measles-like virus from patients with SSPE (see below), this agent was inoculated into ferrets and other laboratory animals. After intracerebral

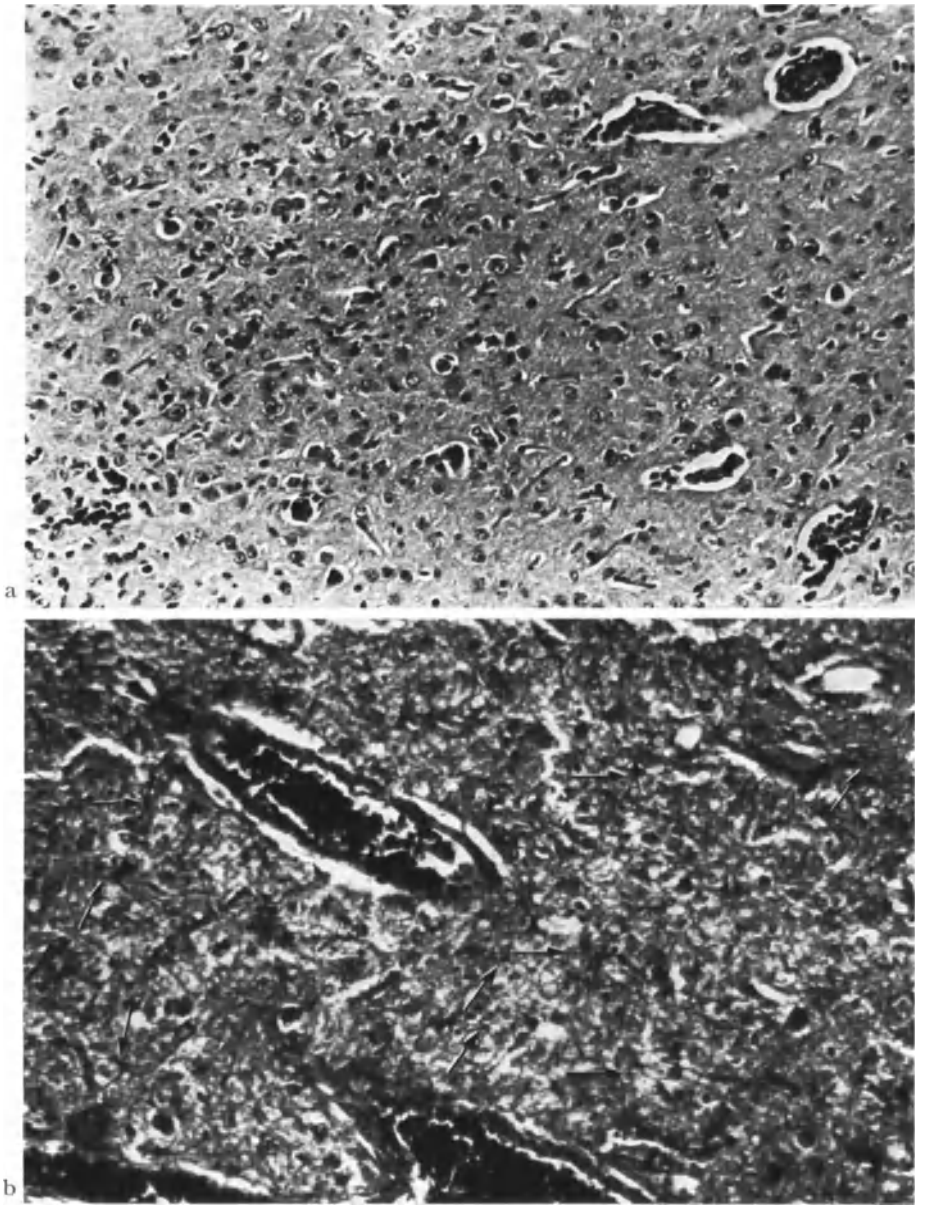



Fig. 13a and b. Encephalitis in ferrets inoculated with human SSPE brain. a) Moderate gliosis and mild perivascular and diffuse lymphocytic infiltration in the basal layer of the cortex in the temporal lobe. H and E stain, ×190. b) Proliferation of fibrous astrocytes (arrows) within the midbrain. Staining with phosphotungstic acid haematoxylin, ×210

inoculation of cell-free virus, the ferrets remained well, and their brains did not show any evidence of encephalitis. However, when either explanted brain cells from SSPE patients or simian tissue culture cells infected with the SSPE measles-like virus were injected intracerebrally into ferrets, the animals

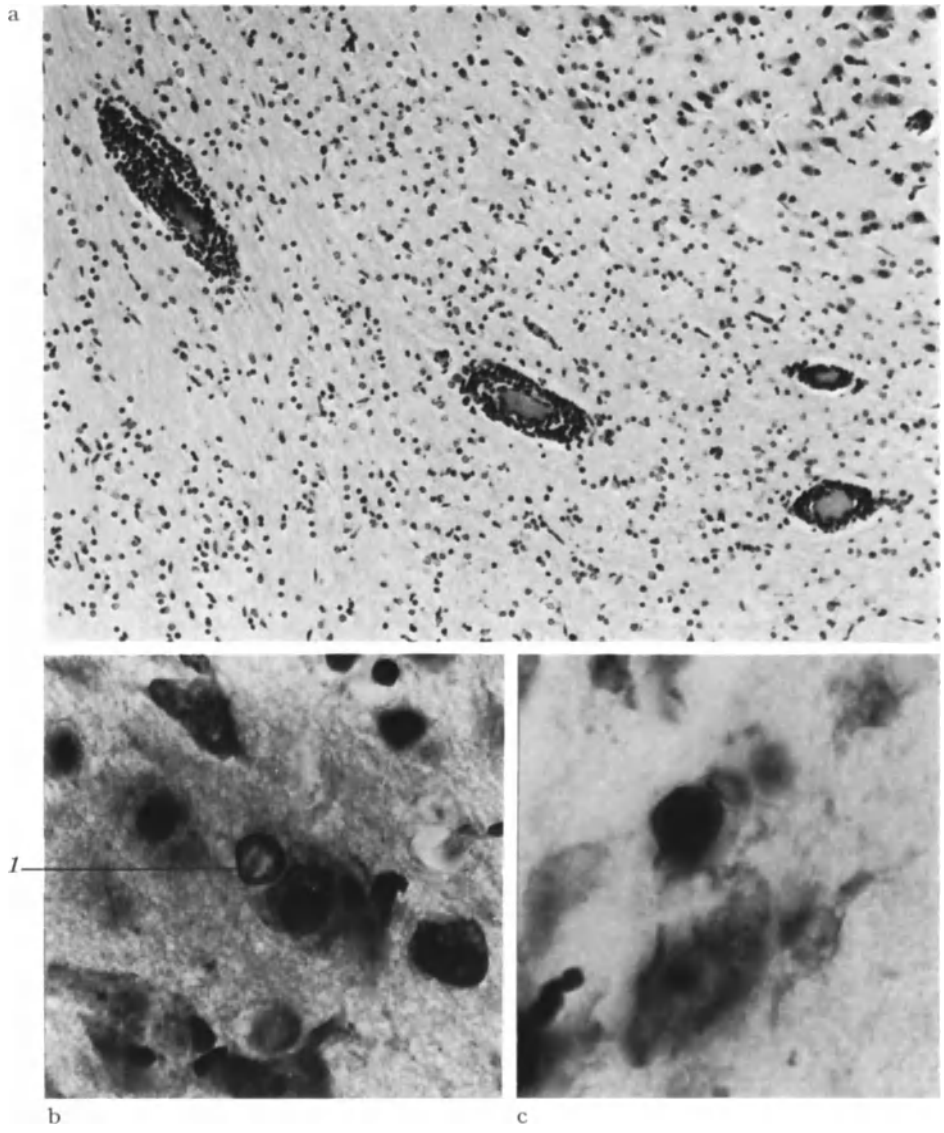



Fig. 14a–c. Encephalitis in ferrets inoculated with SSPE ferret brain, second passage. a) Perivascular cuffing with lymphocytes within the subcortical white matter of the frontal lobe. H and E stain, ×135. b) Nucleoli form type intranuclear inclusion body (*1*) within an oligodendroglial cell. Staining with methylene blue, ×825. c) Intensely stained pyronin positive Cowdry type A RNA-containing intranuclear inclusion body within a glial cell. Methyl green-Pyronine stain, ×1320

developed encephalitis after a relatively short incubation period of two or three weeks, and in these instances the clinical disease was quite striking in most animals. It was characterized by palsy, convulsions and opisthotonos. A histopathological examination of the brain tissue revealed a florrid inflammation, gliosis and numerous Cowdry type A inclusion bodies as demonstrated

in Fig. 14b, c. Some of the inoculated animals, however, showed, at most, only minimal clinical symptoms and yet their brains were no less affected when examined histologically. These animals did show sero-conversion to measles, but the antibody levels remained quite low (KATZ et al., 1970b). Among other laboratory animals that were inoculated, suckling hamsters (LEHRICH et al., 1970) and suckling mice (J. LEHRICH and M. KATZ, unpublished data) developed acute encephalitis when inoculated intracerebrally by cell-free SSPE virus. This suggested that the virus was neuro-adapted and unlike wild measles virus, which produces encephalitis only after adaptation by serial brain passage, was encephalitogenic when recovered from human brain. Adult hamsters, like ferrets, were insusceptible to cell-free virus, but again like ferrets, developed encephalitis when inoculated with tissue culture cells bearing this agent.

*Brain explants.* The technique that was to prove ultimately effective in isolation of the virus was based on the establishment of cell cultures derived from fresh brain tissue. In general, the fresher the tissue, the more successful was the establishment of such cultures. Therefore, tissues obtained at diagnostic brain biopsy yielded the most successful cultures. Autopsy-derived tissues have also been grown, but in our experience a delay of more than six hours post-mortem has usually prevented the establishment of a good culture. The methods used for production of such cultures varied from laboratory to laboratory, but all were essentially dependent on the incubation of small bits of brain tissue in a nutrient medium. The authors have generally refrained from trypsinization of the original tissue and merely minced it with fine scissors and then incubated it at 37°C in Eagle's Basal Medium with 10% fetal calf serum (KATZ et al., 1969b). Others have stressed the importance of initial trypsinization (CHEN et al., 1969; HORTA-BARBOSA et al., 1969b; PAYNE et al., 1969). Once monolayer cultures had been established, serial passage of cells was accomplished in the standard manner by trypsinization.

The cells in such a primary explant culture are quite variable in morphology (Fig. 15) and have not yet been truly defined. They are probably not fibroblasts, because they do not produce fibrin or lay down collagen fibres. Nor do they seem to be glial cells, at least in so far as their staining characteristics are concerned. In the end, by the process of exclusion, one may assume that they are of histiocytic and endothelial origin (TER MEULEN et al., 1970b). After the initial, somewhat tentative growth in culture, these cells usually begin to divide quite rapidly and can be maintained for forty or more generations. A number of such cultures were shown to have the normal human diploid karyotype. In our laboratories two cultures derived from SSPE patients altered their growth pattern by losing contact inhibition and developed an aneuploid karyotype (KATZ et al., 1969a).

In the early split levels, these cells in culture have a fairly uniform appearance (Fig. 16a). In later split levels (fifth to twelfth) they develop a cytopathic effect, first described by BAUBLIS and PAYNE (1968), characterized by the formation of syncytia and giant cells (Fig. 16b, c). These authors, and subsequently others as well, showed by fluorescent microscopy that these cells

Fig. 15. Cells in culture derived from human SSPE brain biopsy material. The cells are of various shapes and sizes and do not exhibit any cytopathic effect (CPE). Methylene blue stain, ×65

contained an antigen reacting with antibodies against measles virus. TER MEULEN et al. (1970a) made the previously noted (see above) differentiation between cytoplasmic and nuclear fluorescence (see Fig. 8). Electron microscopic examination of these cells revealed intranuclear and intracytoplasmic structures resembling paramyxovirus nucleocapsids (KATZ et al., 1969b). Cytochemical studies (TER MEULEN et al., 1970b), parallelling the histochemical ones carried out on the original SSPE brain tissue (MÜLLER and TER MEULEN, 1969) (see above) revealed that, like the brain tissues, these cells contained an accumulation of intranuclear RNA, as well as nucleoproteins, and cytoplasmic RNA. In addition, cytoplasm of three of the cell lines studied in our laboratories contained DNA inclusion bodies (Fig. 18a), not seen in similar cells in cultures derived from non-SSPE brain material (MÜLLER et al., 1971). This finding,

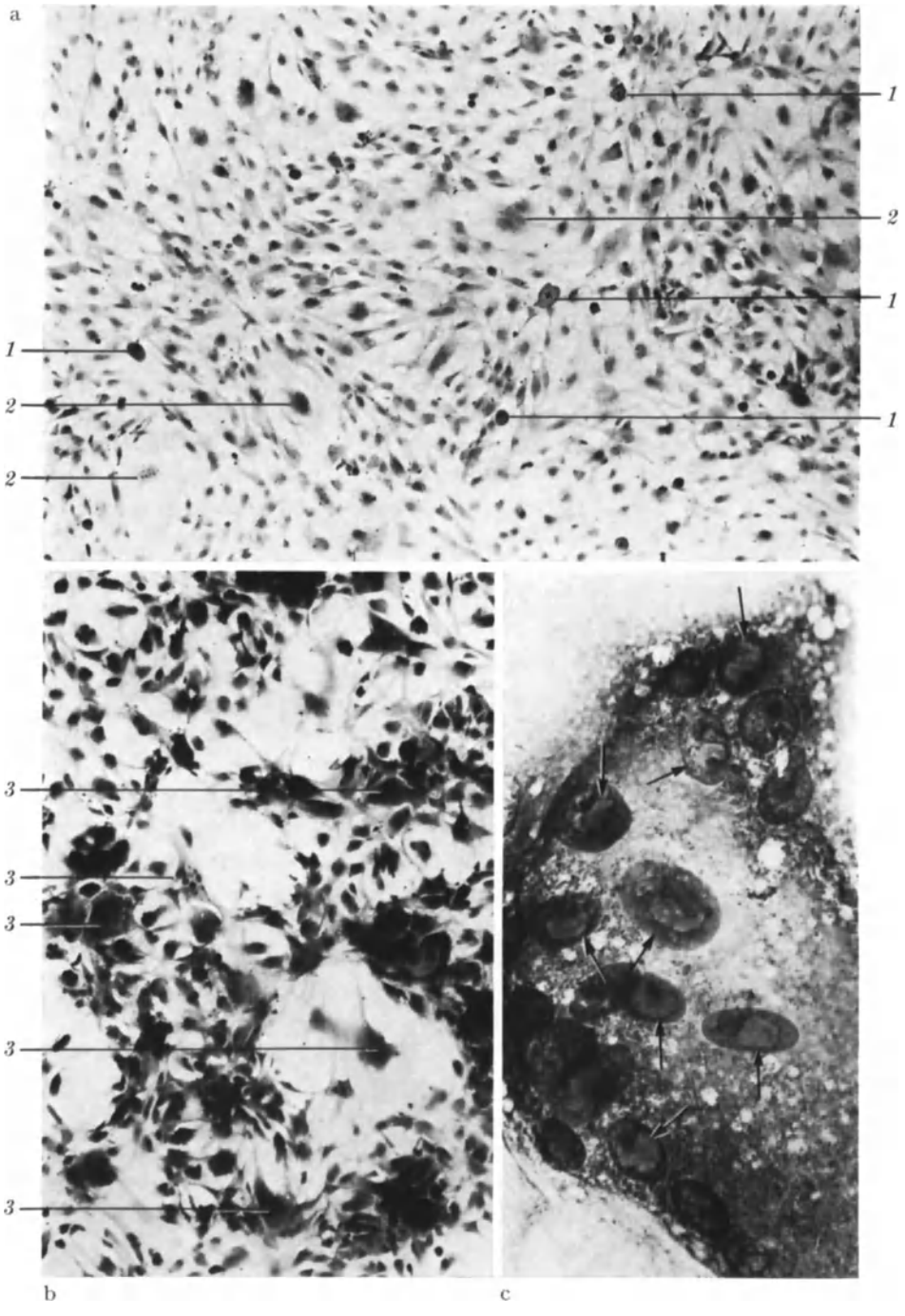



Fig. 16a–c. Cells in culture derived from human SSPE brain biopsy material. a) Cell monolayer showing several mitoses (*1*) and beginning CPE (*2*) ROB, early split level. H and E stain, ×65. b) Intense CPE with formation of syncytia and giant cells (*3*) in ROB, late split level. H and E stain, ×65. c) Multinucleated giant cell with granular and vaculated cytoplasm and pale eosinophilic intranuclear inclusion bodies (arrows). H and E stain, ×600. (Fig. 16c reprinted with permission of TER MEULEN et al., 1970b)

coupled with other evidence from the electron microscopic examination of the isolated viruses (see below), has been taken as evidence suggesting the presence of a second virus in SSPE (Koprowski et al., 1970).

*Isolation of infectious viruses.* The brain cells in cultures derived from SSPE patients seemed to be infected with a virus, because of the progressively increasing cytopathic effect (see above), gradual increment of an antigen reacting with antibodies against measles, development of hemadsorption (Payne et al., 1969), the electron microscopic finding of viral nucleocapsids, and their encephalitogenic potential for ferrets and hamsters. Still, no virus infectious for tissue cultures usually susceptible to paramyxoviruses could be demonstrated in the supernatant medium or in the disrupted cells. It remained for the methods of co-cultivation of the cells with either HeLa (Horta-Barbosa et al., 1969b) or BS-C-1 cells (Payne et al., 1969) and for deliberate cell fusion between the SSPE brain cells and primary AGMK cells (Barbanti-Brodano et al., 1970) to lead to the rescue of infectious viruses. These two methods may not, in fact, differ from each other, since the original brain cell cultures formed syncytia and giant cells that obviously resulted from cell fusion; thus they were endowed with the fusing factor, which was additionally supplied in the form of inactivated Sendai virus in the other method. It must be noted that not every SSPE brain cell culture has yielded infectious viruses. In our laboratories, rescue of the virus has been possible in only two of eight cell cultures, but in these two cultures, rescue could be accomplished repeatedly. Of the six that failed to release a virus, five had a considerably less intense cytopathic effect, little viral antigen, as demonstrated by immunofluorescence, and only a few nucleocapsids; the sixth culture, however, had a very intense cytopathic effect, some viral antigen, and numerous nucleocapsids. In this cell line both the viral antigen and the nucleocapsids vanished with successive passages, but the cytopathic effect became, if anything, more intense.

Once rescued, the viruses were maintained in tissue cultures or stored at −70° C. In general they were all characterized by the production of a cytopathic effect consisting of giant cells, intracytoplasmic and intranuclear inclusion bodies, a reaction with antibodies against measles virus and the induction of antibodies against measles virus in susceptible animals. When examined with the electron microscope, the viral particles were comparable in size to those of measles virus and they were seen budding at the cell surface. Of the five isolated viruses reported (Horta-Barbosa et al., 1969a; Payne et al., 1969; Barbanti-Brodano et al., 1970), four resembled each other closely: the SSPE 2 virus of Horta-Barbosa et al. (1969b), the SSPE virus of Payne et al. (1969) and the JAC and LEC viruses isolated in our laboratories (Barbanti-Brodano et al., 1970). All four viruses grew slowly, tended to produce only low titers of infectious virus, and developed hemagglutinating capacity only with successive passages *in vitro*. The fifth virus, the SSPE 1 virus of Horta-Barbosa et al. (1969a) appeared to be different. It grew rapidly, reached very high titers of infectious virus and produced large amounts of hemagglutinin. A comparative study of the five viruses has not yet been made in one laboratory.

In our laboratories the JAC and LEC (SSPE-derived) viruses have been compared with two strains of measles virus—Woodfolk, a wild strain, and Edmonston, an attenuated strain. Some distinct differences between the SSPE-derived viruses and the measles viruses were noted. The JAC and LEC viruses grew more slowly and yielded less infectious virus than did the measles virus. They produced sharply demarcated plaques, in contrast to the measles virus whose plaques tended to be oblong and lacked distinct borders. Although intracellular viral antigen appeared in the cytoplasm at the same time in cells infected with all four viruses, intranuclear antigen (see above) appeared 48 hours earlier in the SSPE virus-infected cells. The most striking, and as yet not well understood, difference between the two SSPE viruses tested and the measles viruses lay in the susceptibility of SV40-transformed cells and cells derived from non-SSPE-infected brain tissue to the measles, but not to the SSPE viruses. In the case of the SV40-transformed cells this resistance to infection with SSPE viruses may have been related to poor penetration. The transformed cells have been shown to have a highly modified surface (Vorbrodt and Koprowski, 1969) characterized by villi that might interfere with a virus, whose population contains a high proportion of incomplete particles (see below). This hypothesis was supported by the observation that unlike measles viruses, the SSPE viruses, after an initial adsorption to the cell, are released back into the medium. The resistance of the brain cells to the virus remains unexplained. The encephalitogenic potential of the virus for experimental animals, already discussed, also sets them apart from measles virus.

Electron microscope observations of the four viruses also distinguished them. In a study comparing the ultrastructure of CV-1 cells infected with viruses derived from SSPE and that of those infected with measles viruses, Oyanagi et al. (1971) showed that both groups of viruses produced two types of nucleocapsid structures: smooth filaments, 150 to 170 Å in diameter, and granular filaments, 220 to 250 Å. The smooth and granular filaments produced by both types of viruses did not differ in appearance. However, in the cells infected with the SSPE viruses, smooth filaments formed large intranuclear inclusion bodies and granular filaments occupied a large area of the cytoplasm, always sparing the area under the cell membrane. Particles budding from the surface of these cells contained no nucleocapsids (Fig. 17a). In cells infected with measles virus, only small aggregates of smooth filaments were seen in the nuclei. Granular filaments in the cytoplasm predominantly occupied the area under the cell membrane, and were aligned beneath the cell membrane in a parallel fashion and assembled into budding particles (Fig. 17b). These differences, although they were quantitative, distinguished the SSPE from the measles viruses. Moreover, formation of large nuclear inclusion bodies that were filled with smooth filaments appeared to be characteristic of SSPE, but not of measles virus, since this type of inclusion body has been seen invariably in SSPE brain tissues and brain cell cultures and in CV-1 cells infected with SSPE viruses.

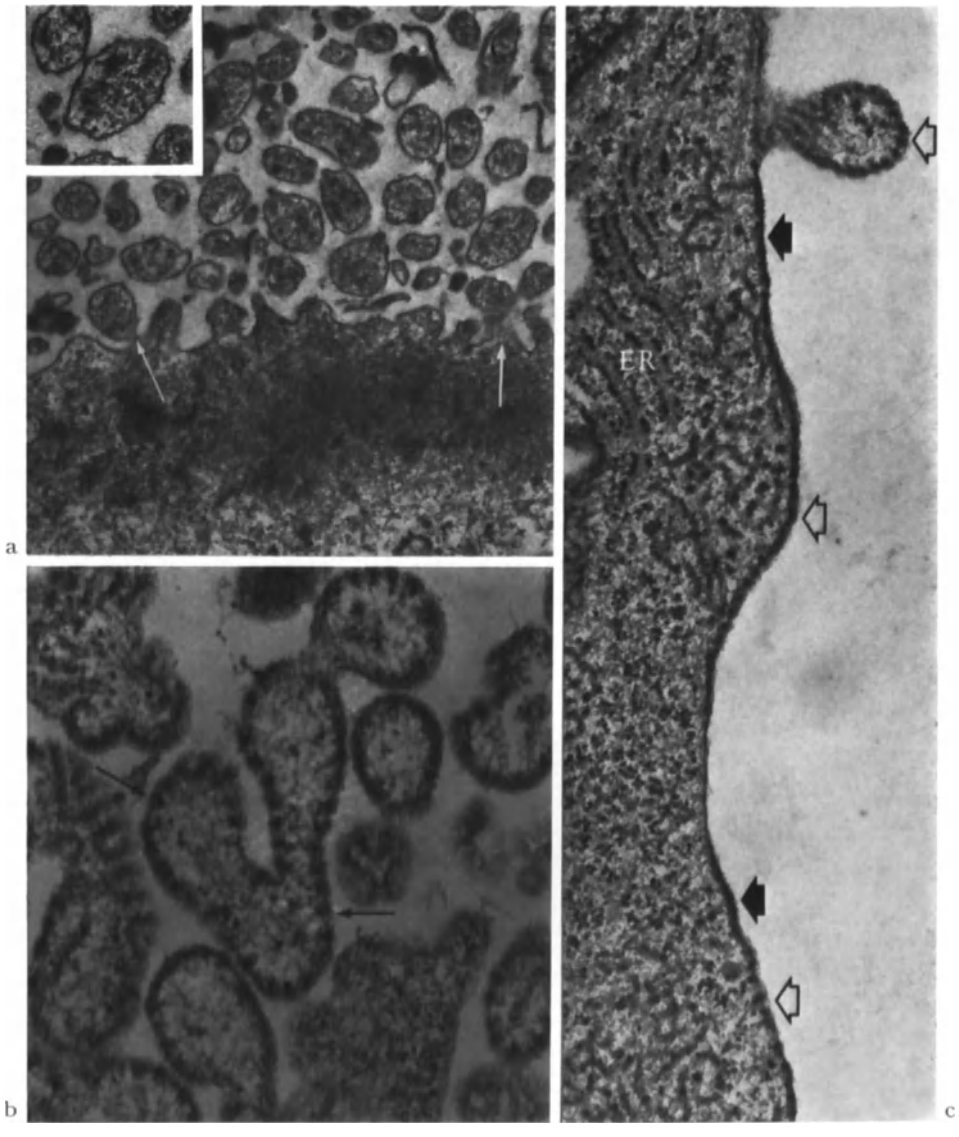



Fig. 17. a) Surface area of a giant cell infected with LEC SSPE-virus. Numerous pleomorphic particles (inset) are seen on the undulated cell membrane. Some particles are being formed through the budding process (arrows). ×17000. Inset ×48000. b) Virions from a CV-1 cell infected with the wild strain of measles virus. Granular filaments are aligned under the envelope of the virions (arrows). ×80000. c) CV-1 cell infected with the wild strain of measles virus. Parallel alignment of granular filaments under the cell membrane and the envelope of a particle in the process of budding (arrows with light heads). Portions of the cell membrane where granular filaments assume the tangential position appear thickened (arrows with dark heads). Minute spikes are projected to the outside from the changed membrane. Note association of granular filaments with the endoplasmic reticulum (ER). ×40000. (Reprinted with permission from Oyanagi et al., 1971)

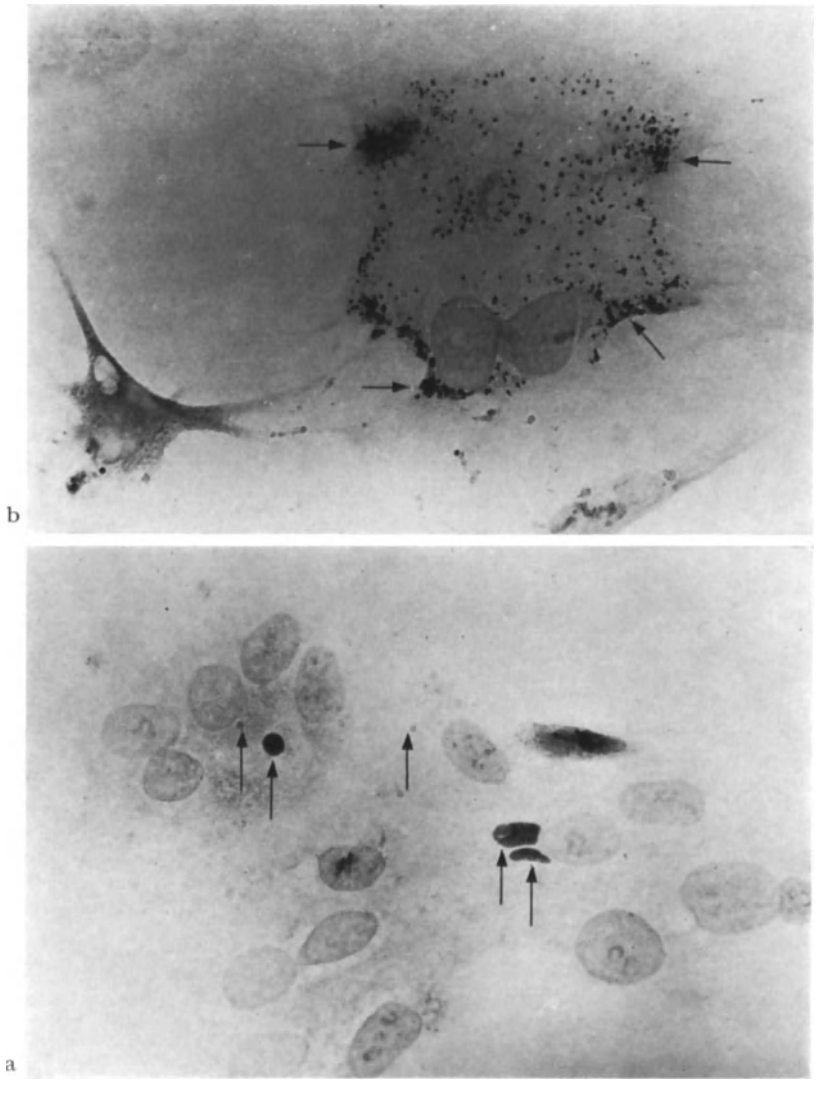



Fig. 18. a Human SSPE brain tissue culture (LEC, late split level). Small giant cell with DNA containing cytoplasmic inclusions (arrows). Feulgen reaction, photograph taken with monochromatic light at 560 mμ. ×560. (Reprinted with permission from MÜLLER et al., 1971.) b) African green monkey kidney cells infected with SSPE virus. Development of cytoplasmic DNA containing inclusions at 72 hrs after infection. Feulgen reaction. ×510

The possibility that a second virus may be associated with SSPE was suggested, not only by the previously mentioned DNA inclusion bodies in the cytoplasm of the brain cell cultures, but also by the observation that such inclusion bodies appear in green monkey kidney cells infected *in vitro* with the isolated SSPE viruses (Fig. 18). The treatment of the green monkey kidney cells with DNA inhibitors during the period of infection with the SSPE viruses

completely prevented the appearance of the DNA inclusion bodies, whereas it did not prevent formation of RNA inclusion bodies or synthesis of the measles-like virus (Müller et al., 1971). Evidence for the presence of the second virus also derives from certain electron microscope observations (Barbanti-Brodano et al., 1970; Koprowski et al., 1970; Oyanagi et al., 1970). In the green monkey kidney cells infected with the SSPE viruses, LEC and JAC, there appear not only the mature virions of the measles-like virus, but also intracyto-

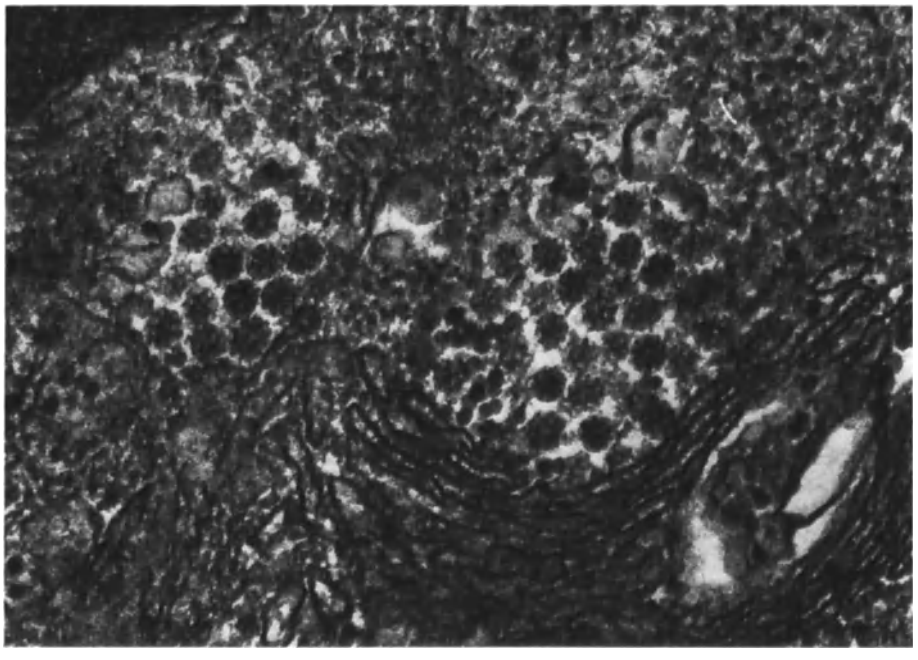


Fig. 19. Papova virus-like particles. They consist of capsomere-like subunits without a limiting membrane. N, nucleus; G, Golgi apparatus. SSPE brain cell culture, 26th transfer level. ×100000. (Reprinted with permission from Oyanagi et al., 1970)

plasmic clusters of virions resembling those of the Papova group. They have been seen in cells infected with several pools of the SSPE viruses. Moreover, such structures were also noted in one of the cell lines (Fig. 19), derived from brain tissue of an SSPE patient, that failed to yield an infectious measles-like virus, as well as in a ferret brain inoculated with the LEC virus. No biological test for the detection of the second virus has thus far been devised.

## VI. Discussion

The demonstration of a measles-like virus in the brain tissues of patients with SSPE and the ultimate isolation by indirect methods of this infectious agent in some cases of this disease have established the relevance of this virus to SSPE. However, the relationship of this virus to the etiology of the disease and to its pathogenesis remains unknown. Indeed, proof that the measles-like

virus is the pathogenic agent is not forthcoming, because Koch's postulates can not be fulfilled. The best approximation of their fulfillment would be the development of an animal model system, wherein this virus, or a wild virus resembling it, such as measles, administered to an animal by an extraneural route, would result, after a long incubation period, in subacute encephalitis. The two experimental studies in animals led to the development of subacute encephalitis in ferrets and acute encephalitis in hamsters and demonstrated the encephalitogenic potential of this virus, but did not lead to a disease that clinically mimicked SSPE.

The agents have not been isolated frequently enough to establish even one of Koch's postulates. Although we do not know how many attempts at such isolation have been made in all laboratories, in our laboratory we were successful in only two out of eight cases, despite numerous attempts in the six cases that did not yield the virus. It is almost as if the failure of isolation, and not the success, were important.

The characteristics of the isolated virus in each case establish it as either identical with, or closely related to measles. Although it has been possible to distinguish the SSPE virus from wild or attenuated measles virus in the authors' laboratory, those differences may not be sufficient to establish the agent as anything but a variant of measles, rather than a separate virus. In the event that the virus is a mutant of measles, one can postulate that an original infection would lead to acute clinical measles, and that the infection might not be aborted, but could smoulder until years later when it appeared as SSPE. It is likely that if such mutants are responsible for SSPE, they would infect not one, but several children in the area where they happen to appear and thus one might expect epidemiologic clusters, or family groups, of SSPE. These have not been demonstrated to everyone's satisfaction, although a claim for their occurrence was made in one study (JABBOUR et al., 1969). If the mutation of measles virus occurs after infection and results from the prolonged residence of the virus in the nervous system, one would expect either that this disease would be more frequent than it is, or if rare, that it would affect only those hosts with a pre-existing abnormality. There is no *a posteriori* evidence that the host is abnormal; of course, it has not been possible to examine these patients *a priori*. In this connection, the hypothesis of BURNET (1968) should be mentioned wherein he suggests that SSPE represents an example of a tolerant infection with measles virus.

A recent hypothesis by BRODY and DETELS (1970) based on the epidemiologic evidence that the disease is predominantly non-urban, with a particular preference for the male sex, has suggested that a zoonotic source might be responsible for SSPE. They considered the possibility that an early measles infection, again supported by the epidemiologic studies, coupled with a superinfection with a second virus—perhaps the previously mentioned papova-like virus—could cause SSPE. It is more likely that exposure to animals would occur in a non-urban setting, even if the animals were the

domestic kind, such as dogs or cats, because in such a locale they are likely to carry agents different from those in their brethren in the cities.

The relationship between the known measles complications, such as encephalitis and giant cell pneumonia, to SSPE remains unestablished. One may wonder whether these represent a different type of an unusual viral involvement of the organs, or else an unusual host response. The suggestion that the latter may play some role is supported by the fact that in the authors' study (Katz et al., 1970a), the antibody responsible for nuclear fluorescence, seen universally in SSPE and acute measles infection, was also seen in two patients who had recovered from giant cell pneumonia. Because of the rarity of these two complications it has not been possible to carry out detailed and comparative studies thus far. Another condition suggestive of an unusual host response to measles is the altered measles reaction in children who had received the killed measles vaccine in the United States and then, when infected with wild measles virus, developed severe clinical illness that often included pneumonia and was associated with unusually high anti-measles antibody titers (Fulginiti et al., 1967). Such patients have not been observed for a long enough period to decide whether they are candidates for SSPE.

It is also possible that SSPE is a result of persistent infection with measles virus, which occurs when this virus infects a patient with partial immunity, either as a result of the persistence of maternal antibody or as a result of waning natural immunity. In this connection one must wonder about the position of the live measles vaccine, vis-à-vis SSPE, because a superinfection —if it were possible—of an immunized individual might lead to SSPE. Before the influence of live measles virus vaccine upon the production of SSPE is assessed, however, a decade or more must elapse.

*Addendum.* Shortly after this chapter was written, three more reports of the recovery of a virus from SSPE-infected brain tissue cultures were published (Kettyls et al., 1970; Parker et al., 1970; Sato et al., in preparation).

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# DNA Replication in Bacteria[1]

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With 6 Figures

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## I. Current Models of DNA Replication

### 1. Chain Elongation

Experiments by CAIRNS (1963), MESELSON and STAHL (1958) and others (see review by BONHOEFFER and MESSER, 1969) have shown that replication of DNA proceeds sequentially, daughter strands of opposite polarity being synthesised concurrently as a replication fork advances along a replicating DNA molecule. On the other hand the bacterial DNA polymerase characterised by

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Kornberg and his collaborators (Kornberg, 1969) as well as those found in phage-infected cells and in the tissues of higher organisms, all appear to utilise 5′ deoxynucleoside triphosphates as precursors and to proceed by chain elongation in the 5′-to-3′ direction only (Richardson, 1969). What then is the mechanism by which the daughter strand that runs 3′-to-5′ with respect to the overall direction of replication is synthesised? One possibility is that its synthesis involves reaction of the 3-OH of deoxynucleoside 5′ triphosphate precursors with a 5′-triphosphate group terminating the growing chain, or reaction of deoxynucleoside 3′-triphosphates with the 5-OH terminus of the growing chain (Mitra and Kornberg, 1966) (Fig. 1 A). However there is no evidence that either type of synthesis can be catalysed by any of the known enzymes, including the recently discovered bacterial DNA polymerase II (see section III), (Richardson, 1969).

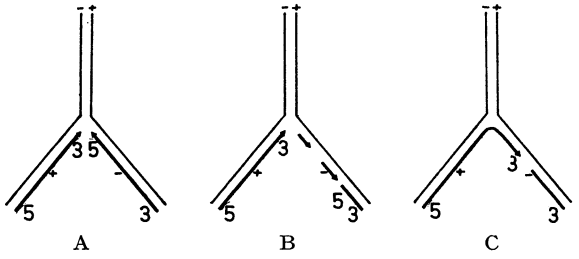



Fig. 1 A–C. Models of DNA chain elongation. A. One strand is synthesised in the 5′-to-3′ direction, the other in the 3′-to-5′ direction. Synthesis could be continuous (as shown) or discontinuous. B. Both strands are synthesised in the 5′-to-3′ direction; the "—" strand is constructed in a discontinuous fashion, the fragments being joined by DNA ligase. The "+" strand could be synthesised either continuously (as shown) or discontinuously. C. The daughter "+" strand loops back periodically onto the other parental template strand. The apex of the loop is nicked and synthesis of the "+" strand continues. Daughter DNA is indicated by darker lines, and the direction of synthesis by arrows

An alternative possibility is that all DNA synthesis occurs in the 5′ to 3′ direction and that the 5-OH-terminated nascent strand is extended by joining to it polynucleotide fragments synthesised "backwards" from the replication fork. Models of this nature have been proposed (Mitra et al., 1967; Okazaki et al., 1968; Gilbert and Dressler, 1968) in which either one (Fig. 1 B) or both strands are synthesised discontinuously. A variant of such models (Fig. 1 C) assumes that the newly synthesised strand loops back from one parental template to the other and that further extension of the fork is initiated by nicking at the apex of the loop (Guild, 1968; Kornberg, 1969; Richardson, 1969; Inman and Schnos, 1971). This scheme is designed to take account of the fact that all DNA polymerising enzymes appear to require the presence of 3′-OH chain termini as primers for synthesis (see Richardson, 1969). Models involving discontinuous synthesis may impose certain constraints on the mechanism of unwinding of parental DNA strands during replication. Provided that at least one daughter strand is synthesised continuously (Fig. 1 B) unwinding could be

driven directly by synthesis; however if both strands are synthesised discontinuously it would have to occur by a separate mechanism. In model C on the other hand unwinding could not take place ahead of the growing fork unless it proceeded in "stop-go" fashion, to permit the switch from one parental template to the other.

Many observations are consistent with the occurrence of discontinuous synthesis. Under normal conditions much of the DNA synthesised during very short times of labelling is recovered after extraction in alkali (or even at neutral pH [OISHI, 1968]) in the form of single-strand fragments of low molecular weight (OKAZAKI et al., 1968) commonly called "Okazaki fragments". After longer periods of labelling progressively more of the labelled DNA recovered is of high molecular weight if the level of DNA ligase activity in the cell is normal, but not if it is reduced (SUGIMOTO et al., 1968; NEWMAN and HANNAWALT, 1968; MASAMUNE and RICHARDSON, 1968). These results suggest but do not prove that "Okazaki fragments" are true intermediates in DNA replication. The possibility that they result at least in part from nuclease action in the replicating region of DNA is underlined by evidence that the yield of fragments depends on whther thymine or thymidine is used to label the DNA (R. WERNER, pers. comm.) and that *nascent* DNA is especially sensitive to degradation when DNA synthesis is interrupted (see p. 57).

Nevertheless a number of observations point directly to a basic asymmetry in the mode of replication of the two daughter strands. OKAZAKI and OKAZAKI (1969 and pers. comm.) obtained evidence by digesting pulse-labelled fragments with specific exonucleases that all phage T 4 DNA synthesis proceeds with 5′-to-3′ polarity; IYER and LARK (1970) and WERNER (pers. comm.) found that no more than half of pulse-labelled *E. coli* DNA was recovered as small fragments, the remainder being in larger variable-sized material, and KAINUMA and OKAZAKI (pers. comm.) found that "Okazaki fragments" in *Bacillus subtilis* hybridise almost exclusively with one of the two DNA fractions which are resolved by "MAK" columns, and so are probably derived primarily from one of the two complementary daughter DNA strands. In addition INMAN and SCHNÖS (1971) have examined replicating phage lambda DNA molecules in the electron microscope and observed that one of the two branches at a replicating fork was frequently connected with the unreplicated parental double-helix by a segment of single-stranded DNA such as might exist during discontinuous synthesis. Taken together these observations indicate that one of the two daughter strands formed at each fork is probably synthesised continuously, the other discontinuously.

## 2. Initiation of Replication

Another aspect of DNA replication that is the object of considerable interest at the present time, concerns the manner in which synthesis is initiated, and, more specifically, the nature of the physical connections between parental and progeny strands during replication. There is good evidence that certain

types of replication involve a rolling-circle intermediate (Fig. 2). Such a structure is derived (GILBERT and DRESSLER, 1968) by nicking one parental strand ("+" strand) of a covalently-closed double-stranded DNA molecule, and somehow moving away the exposed 5′ terminus region so that it is no longer paired with the "−" strand. Once this has occurred the 3′ end of the "+" strand is available as a primer and can be extended by a "conventional" DNA polymerase. The old "+" strand which is progressively displaced from the circular negative strand, can then in turn act as a template for the discontinuous synthesis of a complementary "−" strand. The resulting double-stranded tail

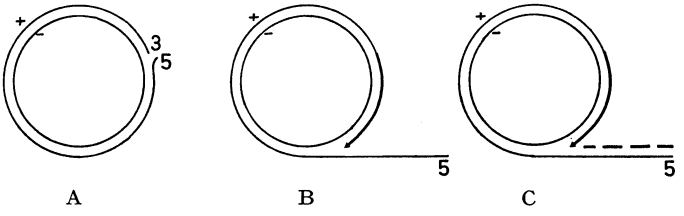
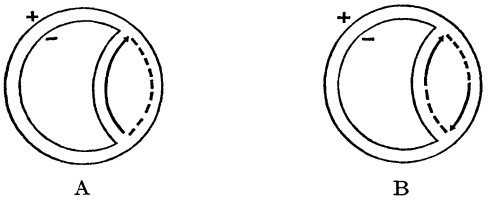



Fig. 2A–C. Rolling circle model of DNA replication. A. The "+" strand of a covalently closed double-stranded circular molecule is nicked (at a unique site) and the exposed 5′ terminus is removed from the negative strand circle. B. The 3′ terminus is extended by a DNA polymerase using the "—" strand as template. This synthesis (heavy continuous line) progressively displaces the 5′-terminal segment of the "+" strand. C. The displaced "+" strand acts as template for the assembly of a complementary "—" strand (heavy dashed lines) by a discontinuous mechanism such as depicted in Fig. 1B or C. Arrow indicates growing 3′ terminus

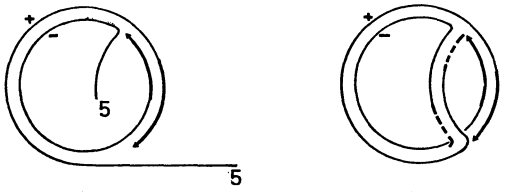
will in principle grow *ad infinitum* and can give rise to circular daughter molecules by recombination between homologous sequences of identical genomes repeated along the tail.

The rolling circle model accounts for the generation of concatenates during the multiplication of phages (FRANKEL, 1968) and for many aspects of single-stranded phage DNA replication (DRESSLER and WOLFSON, 1970, DRESSLER, 1971) as well as for DNA transfer during bacterial conjugation (VAPNEK and RUPP, 1970). However is fails to explain a number of features of other types of replication. Thus replicating molecules frequently occur as unbranched circular structures ("Cairns circles", see Figs. 3 and 4) despite treatment with detergents, pronase and phenol (CAIRNS, 1963; BLEECKEN, STROHBACK and SARFERT, 1966; BODE and MOROWITZ, 1968; KIRSCHNER et al., 1968; TOMIZAWA and OGAWA, 1968; HIRT, 1969). Furthermore in rapidly multiplying cultures of *E. coli* and *B. subtilis*, or after an extended period of inhibition of bacterial DNA synthesis, replication is dichotomous; new rounds are initiated at the replication origin before a previous round has been completed, and when this occurs replication takes place symmetrically at the origin of both daughter chromosomes rather than of only one (QUINN and SUEOKA, 1970; CARO, 1970; BIRD and LARK, 1968). Finally, although there is reason to believe that replication is unidirectional in *B. subtilis* and probably also in *E. coli*, SCHNÖS




A B

Fig. 3A and B. "Cairns" models of DNA replication. The new strands of DNA are not covalently joined to parental DNA. A. Unidirectional replication. Daughter strand synthesis could occur by any of the mechanisms depicted in Fig. 1. Rotation of the parental DNA takes place about a "swivel" at the origin of replication (CAIRNS, 1963) or at one or more discontinuities in the parental DNA strands. B. Bi-directional replication. Synthesis occurs in both directions from the origin; rotation must occur about discontinuities in the unreplicated segment of the chromosome. Arrows indicate growing 3′ termini. Heavy solid lines represent segments that could be synthesised in continuous fashion by a conventional DNA polymerase reaction; dotted lines those that would have to be made discontinuously. Whether replication is undirectional or bidirectional depends on whether one or both 3′ termini of the newly synthesised strands are elongated by continuous synthesis




A B

Fig. 4. Opposing rolling circle model. In this model the initial events in replication are the same as in the basic rolling-circle model (Fig. 2A and B). Thereafter, a second rolling circle is formed by nicking the negative strand almost opposite the site of initiation on the positive strand. Elongation of the newly exposed 3′ terminus displaces a region of the "—" strand which can pair with a complementary sequence on the "+" strand segment already displaced by the first rolling circle. Once this has occurred discontinuous synthesis (dashed lines) fills in the single stranded regions present on either side of the overlap (B). Further elongation at the two 3′ termini (arrows on heavy solid lines) and discontinuous synthesis on the corresponding displaced single stranded regions results in the formation of a double-length circle that can be split by recombination into two unitlength circles (DRESSLER and WOLFSON, pers. comm.; see also WATSON, 1970). The problem of rotation in this model is the same as in the bidirectional "Cairns" model (Fig. 3B)

and INMAN (1970) have recently shown that replication in phage λ occurs in both direction from a single initiation site.

Two models of DNA replication account reasonably well for these features. In one, the "Cairns" model (Fig. 3), daughter strands are not covalently connected to either parental strand; initiation could involve, for example, "priming" by oligonucleotide fragments (BOLLUM, 1964; GOULIAN, 1968). Provided that formation of one strand is initiated, the other can be synthesised by one of the discontinuous mechanisms already discussed. The other model is an extension of the rolling circle model, referred to as the "opposing rolling-circle model" (DRESSLER and WOLFSON, pers. comm., see also YOSHIKAWA, 1967; 1970 and ALBERTS, 1970). It is described in Fig. 4 and its legend. In their

simplest form both models lead to symmetrical bidirectional replication. If, however, extension of one of the two 3′ termini was inhibited, one direction of replication would predominate. Both models require some mechanism to permit the rotation of parental DNA that must accompany replication. Cairns (1963) proposed that the parental strands are connected at the origin and terminus of replication through a device termed a swivel. However there is no evidence for such a structure. Instead rotation may occur at discontinuities in the parental DNA (Hanawalt et al., 1968; Bonhoeffer and Messer, 1969). Such discontinuities would have to be repaired before a replication fork reached them, otherwise they would cause double-strand breakage of daughter chromosomes.

During replication via an opposing rolling-circle structure both strands of *one* daughter arm are synthesised continuously by extension of the 3′ terminus of the parental DNA strands (heavy continuous lines, Fig. 4B) while the strands of the other arm are synthesised in discontinuous fashion, as the parental DNA strands are displaced from the rolling circles (dashed lines). During bidirectional growth on the "Cairns" model the strands which are thought to be extended in a discontinuous fashion are diagonally opposite each other (Fig. 3B). I have already mentioned that Inman and Schnös observed discontinuities at the forks of replicating lambda molecules. Frequently a discontinuity was observed at each of the two branch points of the same molecule, and when this was so the orientation of the discontinuities with respect to one another was as expected on the Cairns model. This result is hard to reconcile with the opposing rolling circle model. On the other hand observations of Yoshikawa (1967; 1970) indicate that there is covalent linkage of daughter DNA strands to parental DNA, and that this is not broken until a subsequent round of replication is initiated. This kind of linkage would be consistent with the opposing rolling circle model but not with the "Cairns" model.

## II. Mutants of DNA Polymerase I

The isolation by De Lucia and Cairns (1969) of a mutant of *E. coli* which appears to lack Kornberg polymerase (henceforth referred to as DNA polymerase I) and shows no striking symptoms other than increased radiation-sensitivity, has greatly stimulated the search for other DNA polymerase activities. Before considering the fruits of this search I shall describe the main features of this and related mutants. The mutation in the strain of De Lucia and Cairns is a recessive amber mutation designated *polA1* (Gross and Gross, 1969). No difference was detected between the behaviour of wild-type DNA polymerase and the enzyme produced in suppressed derivatives of *polA1* (Moses and Richardson, pers. comm.). However, five other *pol*$^-$ mutants with negligible or reduced polymerase activity have been isolated by De Lucia and Cairns (unpubl.); those tested have been shown by mapping and complementation analysis to be in the same gene as *polA1* (Peacey and Gross,

unpubl.), and one of them, *polA6*, has been shown to produce an altered DNA polymerase (KELLEY and WHITFIELD, 1971). We believe therefore that the *polA* locus is the structural gene for DNA polymerase I.

Polymerase-defective mutants of *E. coli* have also been isolated by KATO and KONDO (1970) and ZISSLER (pers. comm.). In addition, after the failure of an extensive search for a thermolabile DNA polymerase I among temperature-sensitive mutants of *B. subtilis* defective in DNA synthesis (GROSS, KARAMATA and HEMPSTEAD, 1968), HEMPSTEAD (1968) isolated and screened ninety mitomycin sensitive mutants and found three which had little if any assayable DNA polymerase. BAZILL (unpubl.) has confirmed the absence of DNA polymerase activity in these strains and has isolated in additional *B. subtilis* mutant which produces a thermolabile DNA polymerase I.

The amber DNA polymerase fragment in the *E. coli polA1* strain appears to have little if any activity *in vitro*. DE LUCIA and CAIRNS found that the supernatant of sonicated preparations of *polA1* cells contained 0.5–1 % of the DNA polymerising activity of the parent strain. This residual activity could be due to "leakiness" of the *polA1* mutation, or to the presence of some molecules of DNA polymerase II (see next section), which according to MOSES and RICHARDSON (1970c) contributes about 5 % of the total DNA polymerising activity of $pol^+$ cells. MOSES and RICHARDSON (pers. comm.) were unable to detect the exonucleolytic activities associated with DNA polymerase I in *polA1* extracts. These workers (1970a) also examined the effect of the *polA1* mutation on DNA polymerase I activity in cells that had been toluene-treated to render them permeable to deoxynucleoside triphosphates. They observed that the repair-like synthesis performed by DNA polymerase I was reduced at least twenty-fold in *polA1* cells (see p. 47).

The isolation of mutants with greatly reduced levels of DNA polymerase I activity is not of course proof that the enzyme does not play an essential role in DNA replication; one can always argue that a very few molecules suffice, or that the activity of the mutant enzyme is greater *in vivo* than under the conditions of assay. All the $pol^-$ strains so far examined appear to have single-site mutations. We have tried without success (CAIRNS and GROSS, unpubl.) to isolate a *polA* deletion mutant by selecting for loss of function of genes near to *polA*, in one case of the *chlB* locus (CASSE, 1970), in the other, of a P2 prophage at location II (CALENDAR and LINDAHL, 1969). Failure could of course be simply due to the presence of one or more indispensable genes in the neighbourhood of the *polA* locus.

The growth of *polA1* cells is essentially normal (MONK et al., 1971; KUEMPEL and VEOMETT, 1970), as is their ability to support the multiplication of all phages tested, including ØX 174, and T 4 (DE LUCIA and CAIRNS, 1969), and to carry out genetic recombination (GROSS and GROSS, 1969; KATO and KONDO, 1970). On the other hand they are more sensitive than wild-type cells to UV irradiation, methylmethane sulphonate (MMS) and X-rays (GROSS and GROSS, 1969; KATO and KONDO, 1970) and show reduced ability to support the growth of U V or X-ray damaged phages (KATO and KONDO, 1970; SMITH, S. M. et al., 1970;

Klein and Niebch, 1971). *polA1* cells are as sensitive to X-rays as *recA*$^-$ cells (Town et al., 1971), less sensitive than *recA*$^-$ cells to MMS (Gross et al., 1971), and substantially less UV sensitive than either *recA*$^-$ or *uvr*$^-$ cells (Kanner and Hanawalt, 1970; Monk et al., 1971). Although *polA1* cells are capable of maintaining most plasmids tested, they are unable to harbour the *colE1* factor, and the *colE2* factor is unstable (Kingsbury and Helinski, 1970). In addition recombination-deficient (*red*$^-$) mutants of phage lambda grow poorly in *polA1* cells (Zissler, pers. comm.), the proportion of ØX 174 replicative form DNA molecules that contain single-strand gaps is greater than normal (Scheckman et al., 1971) and spontaneous mutability appears to be somewhat increased (Coukel and Yanofsky, 1970; Kondo et al., 1971; Gross, unpubl., Witkin, pers. comm.).

These defects probably all reflect a decreased ability to repair single-strand gaps. One can account for their relatively trivial nature in one of two ways: either *polA1* cells retain substantial DNA polymerase I activity, as proposed by Boyle et al. (1970), Kanner and Hanawalt (1970), and Scheckman et al. (1971), or polymerase I activity is to all intents and purposes absent, and the functions that might have been ascribed to that enzyme are performed by other enzymes. I believe that the latter is the correct explanation. It is supported by the work of Moses and Richardson with toluene-treated cells, cited above, and more directly by that of Town et al., who have shown that "rapid repair" of X-ray damage—presumably due to repair by DNA polymerase I of gaps resulting from X-irradiation—does not occur in *polA1* cells. It is also favoured by the discovery of an apparently distinct membrane-bound DNA polymerase (see next section), and by evidence that the *recA* gene product, probably acting in concert with other components, can substitute to some extent for DNA polymerase I in repairing radiation-induced gaps as well as in performing an unknown function necessary for growth.

Let us briefly consider this last point. The existence of an indispensable function that can be performed either by DNA polymerase I or by the *rec* "system" is indicated by the fact that, whereas *polA1* cells and the various types of *rec*$^-$ cells grow reasonably well, the combination of *polA1* with *recA*$^-$ (Gross et al., 1971) and probably also with *recB*$^-$ (Willetts and Gross, unpubl., A. Ganesan, A. J. Clark, M. Oishi, pers. comm.) is inviable. We have suggested that the critical defect corresponds to the repair of gaps which arise "spontaneously" perhaps as a result of discontinuous DNA replication or of nuclease action. The idea that the "*rec* system" can repair gaps is supported by evidence obtained by Monk et al. (1971) that this system rather than DNA polymerase I is responsible for the remarkably efficient repair of gaps produced by dimer-excision in *polA1* cells (Boyle et al., 1970), as well as by evidence for a "delayed" process of repair of X-ray damage dependent on the *recA* gene product (Kapp and Smith, 1970). Since replication of DNA that contains gaps would result in chromosome fragmentation, *recA*-dependent gap-repair may take place in association with the replication fork as it travels along a duplicating DNA molecule. Such a mechanism could repair strand discontinuities that permit rotation of parental DNA during replication (see section I).

KUEMPEL and VEOMETT (1970) have suggested on the basis of quite different results that DNA polymerase I may be involved in joining together fragments produced as a result of discontinuous synthesis. They observed that virtually all the label incorporated into DNA by *polA1* cells during a short pulse was recovered in low molecular weight pieces, whereas in *pol*$^+$ cells a substantial proportion was in large material. When the pulse was followed by a "cold chase" of two to five minutes' duration the molecular weight distribution of the labelled DNA was the same for the two strains. However the experimental procedures employed may not have prevented fragmentation of newly synthesised DNA—as well as repair of the fragments by DNA polymerase I—during lysis of the cells and extraction of the DNA for analysis. The observed fragmentation and repair of nascent DNA by DNA polymerase may therefore not reflect processes actually occurring during DNA synthesis. It should be noted in connection with this alternative interpretation that *pol*$^+$ cells can perform extensive repair of X-ray induced gaps in the cold (TOWN et al., 1971).

## III. DNA Synthesis that is Independent of DNA Polymerase I

### 1. Synthesis in Toluene-Treated Cells

MOSES and RICHARDSON (1970a) observed that cells that have been exposed to low concentrations of toluene are permeable to deoxynucleoside triphosphates and are able to synthesise DNA provided that ATP is also present. They obtained good evidence that this synthesis corresponds to true DNA replication. It proceeds for about an hour at a linear rate comparable to that observed in whole cells, and is semi-conservative. Moreover it was affected at high temperature in two temperature-sensitive mutants (see section VIII). A number of observations indicate that the synthesis does not depend on DNA polymerase I. Thus, the same rate of synthesis is observed in *pol*$^+$ and *polA1* cells, and synthesis is inhibited by sulphydryl-blocking compounds that have little effect on DNA polymerase I, but not by antiserum prepared against the latter enzyme. The range of single-strand molecular weights of the newly synthesised DNA was the same in *pol*$^+$ and *pol*$^-$ cells, but the proportion of pieces of smaller size was greater in the case of *pol*$^-$ cells.

The same workers also detected a type of DNA synthesis that was not semiconservative after exposure of toluene-treated cells to low concentrations of pancreated DNase or to EDTA. This "repair" synthesis does depend on DNA polymerase I since it is reduced at least twenty-fold in *pol*$^-$ cells and is inhibited by antiserum to DNA polymerase I but not by sulphydryl-blocking compounds. It is not ATP dependent, nor is it inhibited in the two temperature-sensitive mutants that were examined

A striking feature of these observations is the marked ATP dependence of "replicative" synthesis. It is unlikely that the ATP is required to protect the

precursors against degradation, or to regenerate them after degradation, since repair synthesis under the same conditions is not ATP dependent. Nor is it required for limited digestion of the template DNA by the ATP-dependent exonuclease coded for by the *recB* gene (BUTTIN and WRIGHT, 1968) since synthesis was shown to be normal in toluene-treated *recB*$^-$ cells. MOSES and RICHARDSON concluded that ATP may either act as a cofactor for the relevant polymerase or be required for some associated energy-dependent reaction. The first explanation has been rendered unlikely by the observation that purified DNA polymerase II, the enzyme which is probably responsible for this synthesis, does not require ATP (see below). It is tempting to suppose that the ATP acts as a source of energy for a process of active unwinding during DNA replication (CAIRNS and DENHARDT, 1968) and this idea receives some support from the observation that ØX 174 single-stranded rings can be converted into double-stranded molecules in the presence of cyanide whereas host DNA replication is inhibited (CAIRNS and DENHARDT, 1968). Synthesis in toluene-treated cells is also strongly dependent on potassium ions (MORDOH et al., 1970). Conceivably this and the ATP requirement reflect the activity of a potassium-dependent ATPase active in unwinding double-stranded DNA.

## 2. Synthesis in "Membrane" Preparations

Semi-conservative DNA synthesis has been detected by several groups of workers in lysates of penicillin- or lysozyme-induced spheroplasts of *polA1* cells (D. W. SMITH et al., 1970; KNIPPERS and STRÄTLING, 1970; OKAZAKI et al., 1970). The bulk of the synthetic activity was associated with the "membrane" fraction which was either sedimented by low speed centrifugation (KNIPPERS and STRÄTLING, OKAZAKI et al.) or embedded in agar (SMITH et al.). The membrane association of this activity is one of the most striking properties that distinguish it from DNA polymerase I. Synthesis was in all cases independent of added primer DNA, strongly inhibited by sulphydryl-blocking compounds and stimulated up to three-fold by ATP. It was not inhibited by nalidixic acid (OKAZAKI et al., 1970). The optimum conditions of pH, ionic strength and magnesium concentration were significanctly different from those for Kornberg polymerase (KNIPPERS and STRÄTLING, 1970). A similar activity could be detected in washed "membranes" from *pol*$^+$ cells.

The membrane activity so far reported is short-lived; synthesis begins to level off after one or two minutes at temperatures between 20° C and 37° C. The reason for this is unclear since pre-incubation in the absence of precursors has little effect on subsequent synthesis (SMITH et al.). Addition of more deoxynucleoside triphosphates (SMITH et al.), or of the supernatant fraction of lysed cells (KNIPPERS and STRÄTLING), did not restore synthesis, and purified Kornberg polymerase added to the membrane preparation showed linear activity for at least thirty minutes (KNIPPERS and STRÄTLING). Synthesis appears to depend at least in part on the integrity of the membrane complex since it was greatly reduced by sonication or by treatment with the detergent "sarkosyl".

However, it was almost unaffected by shearing with a Vortex mixer and therefore presumably does not depend on the integrity of the bacterial chromosome (SMITH et al., 1970).

SMITH et al. isolated the hybrid DNA made during thirty seconds' incubation in the presence of BuTP and found that the largest hybrid pieces had a molecular weight of thirty million. From this they calculated the rate of chain elongation to be $1.5 \times 10^3$ nucleotides per sec, which is similar to the *in vivo* rate and much faster than the synthesis observed with purified DNA polymerase I. The initial rate of synthesis in the system of KNIPPERS and STRÄTLING was of the same magnitude. As in the case of synthesis in toluene-treated cells much of the DNA synthesised in the lysed cell preparations was of low molecular weight, and some of it was single-stranded (KNIPPERS and STRÄTLING; OKAZAKI et al.). The addition of ligase to the reaction did not increase the size of the products (KNIPPERS and STRÄTLING); their low molecular weight may be due to the absence of DNA polymerase I.

Observations with lysates of phage-infected cells support the idea that the synthesis observed in these preparations represents real replication. KNIPPERS and STRÄTLING found that the fraction of synthesis in ØX 174-infected cells which was phage-specific was the same *in vitro* as it was *in vivo*. They also observed that much of the ØX-specific DNA synthesised in vitro when BuTP replaced TTP was hybrid in density, and that about 20 % was fully heavy. Both classes of molecule were biologically active in the spheroplast infectivity assay. However, whereas most of the DNA of the infecting phage retained its closed circular form after extraction from infected cells, all the DNA synthesised *in vitro* was linear. LINNEY and HAYASHI (1970) have detected an additional slow-sedimenting peak of activity in lysates of ØX-infected *polA1* cells that appears to carry out single-strand progeny DNA synthesis. In addition OKAZAKI et al. (1970) have examined DNA synthesis in membrane fractions prepared at various times after infecting *polA1* cells with phage T 4 and have demonstrated a striking parallelism between T 4 DNA synthesis *in vivo* and *in vitro*. The activity of the membrane preparations dropped 10-fold during the first ten minutes after infection with phage T 4 and then returned if the cells had been infected with wild-type T 4 but not if they had been infected with mutants unable to carry out phage DNA replication.

### 3. DNA Polymerase II

An enzyme that can polymerise deoxynucleoside triphosphates has been extracted and purified from *polA1* cells by a number of procedures (KNIPPERS, 1970; KORNBERG and GEFTER, 1970; MOSES and RICHARDSON, 1970b; GINSBERG, PISETSKY and HURWITZ, pers. comm.). It is referred to as DNA polymerase II. The purified enzyme is completely dependent on added primer, shows no ATP dependence, and is inhibited by sulphydryl-blocking agents and 0.2 M KCl, neither of which affect DNA polymerase I. It is not inhibited by antiserum against DNA polymerase I. It appears to be similar in size to DNA

polymerase Ibut differs in its chromatographic behaviour (MOSES and RICHARDSON, 1970c).

KNIPPERS (1970) found that native or denatured *E. coli* DNA of molecular weight about thirty million was a poor primer for DNA polymerase II, as was covalently closed single or double stranded ØX DNA. In addition the enzyme, unlike DNA polymerase I, was unable to commence synthesis at nicks introduced into double-stranded DNA with pancreatic deoxyribonuclease. The only efficient primers were *E. coli* DNA that had been sonicated to pieces of about 1 million molecular weight, and large molecular weight DNA partially digested with exonuclease III. The enzyme thus lacks the nick-translating activity of Kornberg polymerase and appears only able to "fill in" single-stranded regions of otherwise double-stranded DNA molecules by covalent addition to primer DNA presumably at 3-OH termini (KNIPPERS, 1970).

It remains to be proven that DNA polymerase II is the DNA "replicase"; however it is the only DNA polymerising activity detectable in *pol*$^-$ extracts, and it is also present in extracts of wild-type cells (MOSES and RICHARDSON, 1970c; KNIPPERS, 1970) where it is estimated to make up 5% (MOSES and RICHARDSON, 1970c) or 1% (KNIPPERS, 1970) of the total DNA polymerising activity. DNA polymerase II is similar to the T-even phage polymerase (GOULIAN et al., 1968) in that both enzymes lack nick-translating activity, and there is good evidence that the phage enzyme *is* the viral "replicase" since it is absolutely required for phage DNA replication and modification of the enzyme can profoundly effect the fidelity of replication (DE WAARD et al., 1965; SPEYER and ROSENBERG, 1968; DRAKE and ALLEN, 1968). However the discovery of DNA polymerase II has as yet thrown little new light on the mechanism by which semi-conservative DNA replication is achieved; further progress will probably depend on the study of other components of the replication apparatus, such as those responsible for the ATP and $K^+$ dependence of synthesis in toluene-treated cells.

## IV. Mutants of DNA Ligase

The behaviour of mutants defective in DNA ligase is of considerable interest because of the possible role of the enzyme in a discontinuous mode of DNA replication. PAULING and HAMM (1968) have isolated a temperature-sensitive derivative of *E. coli* TAU-bar in which DNA ligase appears to be thermosensitive. It was isolated by the procedure of BONHOEFFER and SCHALLER (1965) adapted in a way designed to enrich for mutants which are unable to grow at high temperature and are also defective in the repair of UV damage at the restrictive temperature. Mutagenised cells were grown at low temperature, starved of required amino-acids and irradiated with UV. They were then transferred to 40° C, incubated in medium containing 5-bromouracil for a short time and illuminated with a sunlamp to kill selectively cells which had carried out repair synthesis at high temperature. This enrichment procedure was repeated twice and samples of the resulting suspensions were plated on rich medium

at 25° C, and replicated to 40° C, to identify temperature-sensitive clones. One such clone had increased UV sensitivity as assessed by incubating cells at 40° C for two hours after irradiation and returning them to 25° C. The final slope of the survival curve for *ts-7* was about twice as steep as that of the parent strain under the same conditions. There was little difference in the sensitivities of parent and mutant cells when plated directly at 25° C.

The DNA ligase of *ts-7* cells has been examined by several workers. PAULING and HAMM (1969) were unable to detect any ligase activity in crude extracts of the mutant. They found about one-fifth of normal activity in partially purified preparations but this activity was not perceptibly temperature-dependent. On the other hand MODRICH and LEHMAN (1971) and GELLERT (pers. comm.) have demonstrated marked temperature-sensitivity of the ligase activity of crude extracts of the mutant assayed in a number of different ways. The temperature-sensitivity is somewhat less marked after partial purification of the enzyme (GELLERT and BULLOCK, 1970; MODRICH and LEHMAN, 1971).

The results of pulse-labelling experiments (PAULING and HAMM, 1969) confirm that *ts-7* cells have a ligase defect; virtually all the labelled DNA recovered from the mutant at 40° was of low molecular weight, even after a pulse of 5 minutes' duration. At their face value these results suggest that all DNA synthesis in *E. coli* is discontinuous and requires ligase to join up low molecular weight fragments. However it remains possible that the fragmentation results from endonuclease action at the growing point, and it may be noted that the idea that synthesis of both daughter strands is discontinuous conflicts with observations discussed in section I.

Examination of the kinetics of macromolecular synthesis in the mutant cells upon transfer to high temperature (MODRICH and LEHMAN, 1971) does not really clarify the role of DNA ligase, and may be complicated by the presence of an inducible phage (IKEDA et al., 1970). Somewhat surprisingly the mutant behaves as though it had a defect in protein synthesis rather than in DNA synthesis: about an hour after transfer cells begin to die and protein synthesis ceases; the rate of DNA synthesis on the other hand increases approximately tenfold during the first hour, as in the parent strain, and thereafter remains constant for at least two more hours. Much of the DNA synthesised in the course of an hour at high temperature in the presence of bromouracil is of a density intermediate between hybrid and light (PAULING and HAMM, 1968) suggesting that a substantial proportion of the synthesis that takes place is some kind of repair synthesis. This would be expected if the absence of functional ligase results in the accumulation of strand interruptions that act as priming sites for, say, DNA polymerase I. The physiological abnormalities of *ts-7* cells and their ligase defect appear to be due to a single mutation since three independent "revertants" of *ts-7* isolated at high temperature were normal with respect to ligase activity and the other properties described above (PAULING and HAMM, 1970; MODRICH and LEHMAN, 1971).

Ligase mutants of *E. coli* K 12 have been isolated by GELLERT and BULLOCK (1970). They first selected a mutant which could support the growth of

ligase-defective strains of phage T 4 and found that it contained approximately five times as much ligase as the parent strain and that the enzyme produced was indistinguishable from wild-type enzyme. Three mutants with *less* than normal ligase activity were then obtained from this "overproducing" strain by selecting derivatives that were *unable* to support the growth of the phage mutants. The ligase defect of the mutants, which were isolated at 37° C, was in each case most pronounced when assayed at high temperatures and varied between about three and ten per cent of wild-type levels at 42°. The ability of the mutant enzymes to form the intermediate enzyme complex with AMP was virtually normal. The enzyme of mutant *lig-4*, which had the lowest activity at 42° C and almost normal activity at 20° C, retained its temperature sensitivity after extensive purification. All three mutants grew normally at 42° C but showed two or three-fold greater UV sensitivity than wild-type at this temperature; at 44° C *lig-4* grew well in a supplemented minimal medium but its growth in tryptone broth was somewhat defective; snakes were formed and there was a loss of viability. Pulse-labelled experiments with *lig-4* showed normal conversion of "Okazaki" fragments to high molecular weight DNA at 42° C.

The results obtained with the *ts-7* mutant of PAULING and HAMM indicate that ligase is an indispensable enzyme, whereas the properties of the mutants of GELLERT and BULLOCK suggest that it may be dispensable. Although its precise function in DNA replication is unclear, it seems to me probable that the former view is correct and that the mutants of GELLERT and BULLOCK are less defective under *in vivo* conditions than they are *in vitro*. This interpretation is supported by the observation that, unlike the *ts-7* mutant, they have almost normal activity when assayed for the formation of the intermediate complex of enzyme with AMP, and it is not inconsistent with the fact that they fail to support the growth of phage T 4 since growth of the phage is affected by quite small variations in level of ligase activity (GELLERT and BULLOCK, 1970). It should therefore be possible to obtain mutants like *ts-7* by further application of the technique of GELLERT and BULLOCK. The fact that the *ts-7* mutant does not show preferential inhibition of DNA synthesis at restrictive temperature makes it hard to understand why the procedure used in its isolation was successful. However it may explain why no thermosensitive DNA-defective mutants have been found to have an altered ligase (see section V). Genetic analysis of the ligase mutations has not yet been reported.

## V. General Properties of Temperature-Sensitive Mutants Defective in DNA Synthesis

Numerous workers have isolated and characterised temperature-sensitive mutants of *E. coli* whose primary defect is in DNA synthesis. Some were obtained by selecting cells unable to incorporate bromouracil (BONHOEFFER and SCHALLER, 1965) or $H^3$-thymidine (FANGMAN and NOVICK, 1968) into DNA at high temperature; others by screening large numbers of temperature-sensitive

Table 1. *dna mutations of E. coli K 12*

| *dna* allele Number | Linkage group | Previous designation | Source reference | Reference for map position |
|---|---|---|---|---|
| 1 | C | dna-301 | CARL (1970) | 2, 7 |
| 2 | C | dna-302 | CARL (1970) | 2, 7 |
| 3 | G | dna-303 | CARL (1970) | 2 |
| 5 | A | dna-305 | CARL (1970) | 2 |
| 6 | B | dna-306 | CARL (1970) | 2, 3 |
| 7 | D | dna-307 | CARL (1970) | 2, 7 |
| 8 | B | dna-308 | CARL (1970) | 2, 3 |
| 21 | B | — | FANGMAN and NOVICK (1969) | 2 |
| 22 | B | tsm-8 | FANGMAN and NOVICK (1969) | 2 |
| 27 | B | ts 27 | INOUYE (1969) | 2 |
| 28 | C | — | SCHUBACH and DAVERN (1971) | SCHUBACH and DAVERN |
| 42 | B | $DNA^{ts}_{42}$; T 42 | HIROTA et al. (1968a) | 1, 4 |
| 43 | B | $DNA^{ts}_{43}$; tsm-4 CR 34 tsDNA; BT 43; T 43 | BONHOEFFER (1966) | 1, 4, 5 |
| 46 | A | T 46; $DNA^{ts}_{46}$ | HIROTA et al. (1970) | 4, 8 |
| 47 | A | — | KUEMPEL (1969) | 6 |
| 59 | B | BT 59 | RICARD and HIROTA (1969) | 1 |
| 70 | B | HfrH 165/70 ts-DNA | LANKA and SCHUSTER (1970) | 2 |
| 83 | A | T 83 | KOHIYAMA et al. (1963) | 4, 8 |
| 101 | F | — | WECHSLER and GROSS (1971) | 2 |
| 107 | B | — | WECHSLER and GROSS (1971) | 2 |
| 125 | B | — | WECHSLER and GROSS (1971) | 2 |
| 126 | — | — | MURGOLA and ADELBERG (1970) | MURGOLA and ADELBERG |
| 142 | B | T 142 | KOHIYAMA (1968) | 1, 4 |
| 173 | B | — | WECHSLER and GROSS (1971) | 2 |
| 177 | A | — | WECHSLER and GROSS (1971) | 2 |
| 194 | | — | WECHSLER and GROSS (1971) | 2 |
| 252 | A | HfrH 252 ts-DNA | LANKA and SCHUSTER (1970) | 2 |
| 266 | B | T 266; $DNA^{ts}_{266}$ | BUTTIN and WRIGHT (1968) | 1, 4 |
| 279 | B | — | WECHSLER and GROSS (1971) | 2 |
| 293 | E | — | WECHSLER and GROSS (1971) | 2 |
| 308 | G | tsm-3 | MARINUS and ADELBERG (1971) | 2, 5 |
| 313 | B | BT 313; tsm-5 | RICARD and HIROTA (1969) | 1, 5 |
| 325 | C | — | WOLF (1971) | WOLF |
| 368 | B | — | WECHSLER and GROSS (1971) | 2 |
| 391 | B | — | WECHSLER and GROSS (1971) | 2 |
| 399 | G | tsm-6 | MARINUS and ADELBERG (1971) | 2 |
| 420 | B | BT 42 | RICARD and HIROTA (1969) | 1 |
| 437 | — | — | BONHOEFFER (pers. comm.) | — |
| 454 | B | ts DNA 454; BT 54 | RICARD and HIROTA (1969) | 1, 5 |
| 486 | E | — | WECHSLER and GROSS (1971) | 2 |
| 500 | B | BT 500; tsm-1 | RICARD and HIROTA (1969) | 1 |
| 508 | A | — | MONK, PEACEY and GROSS (1971) | 2 |

Table 1. (Continued)

| *dna* allele Number | Linkage group | Previous designation | Source reference | Reference for map position |
|---|---|---|---|---|
| 511 | E | — | WECHSLER and GROSS (1971) | 2 |
| 517 | A | — | WECHSLER and GROSS (1971) | 2 |
| 806 | — | — | KOHIYAMA (1968) | — |

The mutations will be referred to as *dna-1*, *dna-2* etc. in conformity with the recommendation of DEMEREC et al. (1966). This designation may also be applied to other kinds of conditional mutants, such as streptomycin-dependent mutants. The list includes one mutant of the latter kind, *dna-126*. Where a mutation has been referred to previously by a designation different from the one employed here, the previous designation is given in the second column of the table. The group assignments correspond to map position (see Fig. 5) not complementation groups, and I suggest that they be placed after the allele number viz. *dna*-1C, *dna*-6B etc. until complementation data are available. The references listed in the Table are not necessarily the first relevant publications, but they are the source of statements in the text that are not otherwise acknowledged.

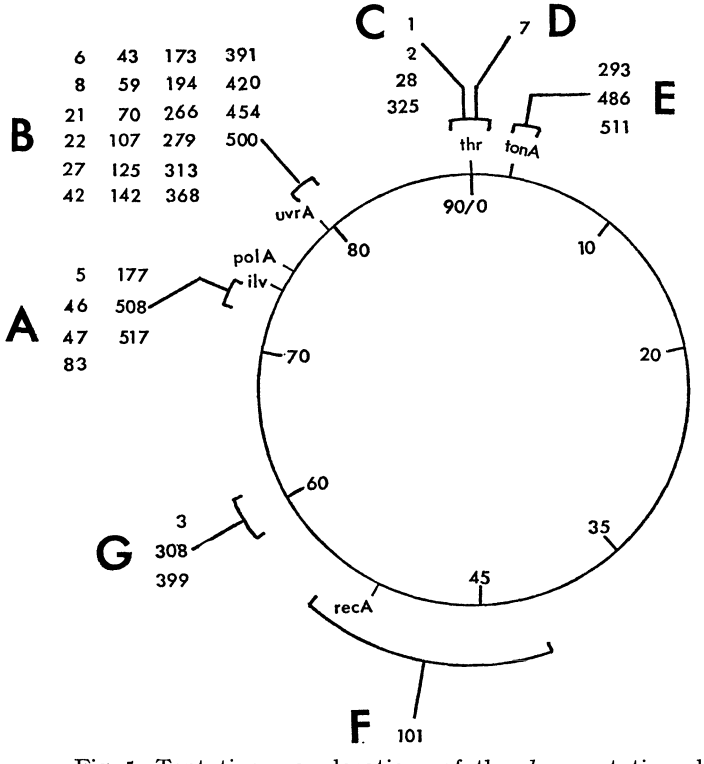
*Abbreviations*: 1 = RICARD and HIROTA, 1969; 2 = WECHSLER and GROSS, 1971; 3 = CARL, 1970; 4 = HIROTA et al., 1968a; 5 = MARINUS and ADELBERG, 1971; 6 = KUEMPEL (pers. comm.) 7 = MONK, (pers. comm.) 8 = HIROTA et al., 1970.

mutants for defective DNA synthesis at high temperature (KOHIYAMA et al., 1966). Dr. J. WECHSLER and I have mapped fifteen mutants isolated in this laboratory by the latter procedure as well as a number of mutants generously provided by other workers. Table 1 lists most of the *dna* mutants of *E. coli* K 12 known to us, and Fig. 5 gives their approximate map positions based on Hfr and F-prime crosses, and P 1 transductions, performed in numerous laboratories. The mutations have been mapped with quite different degrees of precision and some of the groups shown in Fig. 5 will probably be subdivided by more accurate mapping, and by complementation analysis. The information which I shall cite is derived from the references listed in Table 1 unless otherwise acknowledged. Most of the available information on *dna* mutants of *B. subtilis* has been summarised by GROSS et al. (1968) and KARAMATA and GROSS (1970).

A *dna* mutant could be affected in any of three processes: precursor synthesis, initiation of replication or chain elongation. Mutants in groups A and C appear to belong in the second category (see section VII, 2). The remaining mutants are blocked in precursor-synthesis or in replication *per se*. In many of them DNA synthesis ceases immediately upon transfer to restrictive temperature. In others, some residual synthesis takes place, probably because it takes some time for the defective component to be inactivated. In this section I will survey what little is known of the general properties of all these mutants. Certain aspects will be considered in more detail in later sections.

### *Precursor synthesis*

None of the *E. coli* mutants that have been examined are defective in synthesis of deoxynucleoside triphosphates at restrictive temperature. Those




I here present the A, B, C
(And other letters, as may be)
Which spell in a symbolic way
The manner whereby DNA
Of replicons can reproduce—
And other matters so abstruse
That I must have been quite myopic
To choose them as a research topic.
I wish I knew what it all meant,
And could with confidence present
The function which each gene displays
Which nickase, ligase, replicase.
But if these things I truly knew
There'd be no need for this review.

W. H.

Fig. 5. Tentative map locations of the *dna* mutations listed in Table 1. Most of the mutations assigned to group A (HIROTA et al., 1968a) have been shown to be co-transducible with mutations in the *ilv* cluster (HIROTA et al., 1970; WECHSLER and GROSS, 1971) and are located at about 73′ on the standard map (TAYLOR, 1970). Most of the mutations in group B (HIROTA et al., 1968a) have been shown to be co-transducible with the *malB* locus; they are all provisionally located immediately clockwise from *uvrA* (MORDOH et al., 1970). *dna*-21 B and -22 B were previously incorrectly assigned to a position near *str* (FANGMAN and NOVICK, 1968). Group C mutations *dna*-325 (WOLF, pers. comm.) and *dna*-1 and -2 (MONK and GROSS, unpubl.) are linked in transduction with *dra* (89.5′), while the group D mutation *dna*-7 is linked to *thr* (KINROSS and MONK, pers. comm.) and group E to *tonA* (WECHSLER and GROSS, 1971). *dna*-101 F lies within the interval 41′–55′, and *dna*-3 G in the segment from approximately 60′ to 63′ carried by F-prime factor 102 (WECHSLER and GROSS). The streptomycin suppressible mutation *dna*-126, listed in Table 1, has been located between 88′ and 1′ (MURGOLA and ADELBERG, 1970) and may be in group C or D. W. H. = WILLIAM HAYES

examined include several group B mutants (e. g. BONHOEFFER, 1966; FANGMAN and NOVICK, 1968), *dna*-7 D and three initiation mutants (*dna*-325 C-2 C and 47 A). In addition KARLSTRÖM (pers. comm.) has screened all the *dna* mutants isolated in this laboratory (belonging to groups A, B, E and F) for a defect in ribonucleotide reductase activity, with negative results. Thermosensitive mutants of *B. subtilis* have been identified that *are* defective in the reduction of ribonucleotides (BAZILL and KARAMATA, in prep.).

### *Repair synthesis*

Several group B mutants have been shown to carry out normal repair replication in response to UV irradiation at high temperature (BONHOEFFER,

1966; Couch and Hanawalt, 1967). The defect in these mutants is therefore not in a function also required for repair synthesis. Other mutants have not yet been examined.

### *DNA Polymerase I Activity*

Many workers have examined the DNA polymerase I activity of *dna* mutants without finding any with a defect in this enzyme. The mutants tested[3] include representatives of groups A, B and G of *E. coli*, and of at least five groups of mutants in *B. subtilis* (Gross et al., 1968). As discussed in section II, it seems probable that DNA polymerase I is dispensable.

### *DNA Ligase Activity*

At least 22 mutants of the Bonhoeffer collection (see footnote) have been tested and found to have normal DNA ligase activity (Gellert et al., 1968; Schaller, quoted in Bonhoeffer and Messer, 1969; see also Buttin and Wright, 1968). I have already discussed the DNA ligase mutants that have been isolated by specially designed procedures.

### *Loss of Viability*

*dna* mutants of group A and group B, and probably all others, die after prolonged exposure at restrictive temperature. The cause of death is unclear, but it depends on continued metabolic activity (Fangman and Novick, 1968; Buttin and Wright, 1968; Lanka and Schuster, 1970; Carl, 1970) and is probably analogous to thymineless death. Fangman and Novick isolated an interesting derivative of *dna*-22B which failed to die at restrictive temperature but retained the other properties of the mutant.

### *Reversibility*

Many *dna*B mutants recommence DNA synthesis more or less immediately upon return to low temperature after a limited period at restrictive temperature (Inouye, 1969; Ricard and Hirota, 1969). In all those that have been tested recovery occurs in the absence of protein synthesis (Kogoma and Lark, 1970; Worcel, 1970). I will discuss the behaviour of initiation-defective mutants later.

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[3] Mutants tested include *dna*-43B and 70B (Bonhoeffer, 1966), -266B (Kohiyama and Kolber, 1970), -21B, 22B, 27B, 83A, and many representatives of the Bonhoeffer collection of *dna* mutants (Bonhoeffer and Messer, 1969). The collection originally contained several hundred mutants isolated by the enrichment procedure of Bonhoeffer and Schaller (1965). Most have since been discarded and only about a dozen, generally the least "leaky" have been retained. Of these 11 are listed in Table 1. The mutants of that collection that have been mapped fall into groups A, B, or G (see Fig. 5 and Table 1).

### *DNA degradation*

Degradation of preformed DNA has been observed in mutants of groups B, D and G when replication is stopped by transfer to restrictive temperature (BUTTIN and WRIGHT, 1968; CARL, 1971; PEACEY and GROSS, unpubl.). It is also observed when replication is prevented by other means (HANAWALT and BREMPELIS, 1967). No breakdown has been detected in initiation-defective mutants (HIROTA et al., 1968b; CARL, 1970) presumably because replication is not arrested in progress. There is evidence that degradation depends at least in part on the ATP-dependent nuclease specified by the *rec*B gene (BUTTIN and WRIGHT, 1968) which can act both as an exonuclease and an endonuclease (GOLDMARK and LINN, 1970). RASMUSSEN (1968) and MIKOLAJCZYK and SCHUSTER (1971) have demonstrated that breakdown in *dnaB* mutants takes place preferentially in the region of the growing point and there is evidence that only newly synthesised DNA strands not parental strands are degraded (RASMUSSEN). If degradation involves endonucleolytic as well as exonucleolytic cleavage it could lead to fragmentation of pulse-labelled DNA, and so confuse the results of experiments designed to analyse the mechanism of DNA chain elongation, and the role of specific enzymes in that process (see sections II and IV).

### *Cell Surface and Membrane Alterations*

Changes in the electrophoretic pattern of "membrane" proteins have been detected after incubation of a number of *dnaB* mutants at restrictive temperature (INOUYE and GUTHRIE, 1969; INOUYE and PARDEE, 1970; SICCARDI et al., 1971). It is not yet clear whether the changes are indirect consequences of the arrest of DNA synthesis, or whether the *dnaB* locus exerts direct control on the structure or activity of the altered components. On the one hand similar changes have been observed at high temperature in initiation-defective mutants of group A (SHAPIRO et al., 1970) and in *dna*$^+$ cells in which DNA synthesis has been arrested by treatments such as thymine-starvation (INOUYE and PARDEE, 1970; SICCARDI et al., 1971). On the other hand SICCARDI et al. observed the same membrane protein changes when a salt-reversible *dnaB* mutant (see below) was exposed to high temperature under reversing conditions where presumably DNA synthesis was normal.

HIROTA et al. (1970) have reported alterations of cell surface properties that appear to be specifically associated with the arrest of initiation. They observed that *dna*-46A and -83A cells became abnormally sensitive to lysis by deoxycholate after about an hour at high temperature and bound an aniline dye with unusual efficiency. Neither of these changes occurred in *dnaB* mutants or after thymine starvation of *dna*$^+$ cells.

### *Cell Division*

When DNA synthesis is blocked by thymine starvation or by many other treatments *E. coli* cells elongate and do not divide further except for the resid-

ual divisions that segregate already completed chromosomes into separate cells. This is one of the observations which has led to the idea that division is triggered by the completion of rounds of DNA replication (see HELMSTETTER, 1969). In general observations on cell division in *dna* mutants at high temperature are consistent with this view (HIROTA et al., 1968a, b; INOUYE, 1969). Some *dna* mutants nevertheless continue to divide at high temperature and give rise to anucleate cells which may either be of fairly uniform size (HIROTA et al, 1968b) or of variable size (INOUYE, 1969). However division is less efficient than usual so that after some time long filaments are produced. HIROTA et al. (1968a) believe that some, possibly all, of the *dna* mutants that continue to divide carry a second mutation that is responsible for this behaviour.

### *Phenotypic Reversal*

RICARD and HIROTA (1969) reported that DNA synthesis proceeded normally at restrictive temperature in medium containing a high salt concentration, in four out of nine *dna-B* mutants that they examined. They also observed suppression of the inhibition of cell division in a thermosensitive cell-division mutant by high concentrations of various salts and of glucose, but not by glycerol.

Prompted by these observations we have examined the effect of high concentrations of salts or sugars on a large number of *dna* mutants (PEACEY and GROSS, unpubl.). We found that all group B mutants examined (nineteen in all, including four of the five reported by RICARD and HIROTA not to be reversible) as well as all group E mutants could be phenotypically reversed by high glucose concentrations, and that glycerol was also effective in causing reversal. Group A mutants appeared to be inhibited by high salt concentrations even at low temperature, but many were phenotypically reversed by high sugar concentrations. No phenotypic reversal was observed with any mutants of groups D, F and G or with *dna*-1 C and -2 C. Sensitivity to phenotypic reversal is not restricted to mutants of DNA replication; HAWTHORNE and FRIIS (1964) observed that many temperature-sensitive auxotrophs of yeast were reversed by the same agents that subsequently have been found to be effective in the case of *dna* mutations.

### *Recombination Proficiency*

All the *dna* mutants give rise to *dna*$^+$ recombinants when mated with suitable Hfr strains and plated directly at high temperature (a few have not been tested). This does not mean that the mutants are recombination-proficient at restrictive temperature since a recombination deficiency, if it existed, could be complemented by the *dna*$^+$ gene from the donor cell. JOSHI and SIDDIQI (1968) have tested one mutant in a way that avoids this complication. They performed a mating involving a *dna*-43 B recipient, in which the Hfr and the recipient cells carried mutations at different sites in the structural gene for

alkaline phosphase (*pho*$^-$) and the Hfr only rarely transferred the *dna*$^+$ gene, and showed that *pho*$^+$ recombinants were formed with very high efficiency at restrictive temperature by assaying for production of alkaline phosphatase in the zygotes.

## VI. Synthesis of Phage DNA, and Conjugal DNA Transfer, in *dna* Mutants

### 1. Synthesis of Phage DNA

Examination of the effect of host *dna* lesions on replication of the DNA of a variety of phages may throw light on the nature of the defective *dna* functions. T-even phages have been found to multiply at restrictive temperature in all *dna* mutants tested, whereas phage lambda can multiply in initiation mutants (groups A and C), but not in mutants of groups B, D, or G (FANGMAN and NOVICK, 1968; HIROTA et al., 1968a; INOUYE, 1969; FANGMAN and FEISS, 1969; LANKA and SCHUSTER, 1970; CARL, 1970). (Mutants of groups E and F have not been tested.) The difference between the phages is consistent with the fact that lambda codes for many fewer proteins required for its own replication than does a T-even phage, and therefore must depend more on host functions. Phage P1 has been shown to multiply in *dna*-70B and -46A, and replication of the phage DNA can be detected in *dna*-252 cells after host synthesis has come to a halt even though few progeny phage are produced (BEYERSMANN and SCHUSTER, pers. comm., LANKA and SCHUSTER, 1970).

The single-stranded DNA phages ØX 174 and M 13 are unable to multiply in *dnaB* mutants at restrictive temperature (STEINBERG and DENHARDT, 1968; SINSHEIMER et al., 1968; PRIMROSE et al., 1968). The circular single-stranded phage DNA molecules penetrate the host cell but fail to be converted to the double-stranded parental replicative form (RF). If the cells are incubated at low temperature for a short time to permit this step to occur and then transferred to high temperature progeny phage are still not produced, indicating that some later stage of replication is also blocked. However if M 13-infected cells are incubated at low temperature until mature phage particles are being formed and then shifted to high temperature they continue to produce progeny single-stranded DNA and infectious phage for at least two hours (PRIMROSE et al., 1968). ØX-infected cells treated in the same way cease synthesis within a few minutes (STEINBERG and DENHARDT); the difference may be somehow related to the fact that ØX requires many more phage-coded products in order to synthesise progeny DNA than M 13, which requires only one (PRATT, 1969). I will discuss the possible significance of these results below.

### 2. DNA Transfer in Bacterial Conjugation

During transfer by Hfr or F-prime donor cells a single strand of the donor DNA is displaced into the recipient cell while a new copy is synthesised and

retained in the donor cell (see VAPNEK and RUPP, 1970). MARINUS and ADELBERG (1971) have examined F-prime transfer between pairs of donor and recipient cells carrying the same *dna* mutation; six of the mutations whose effect was studied belonged to group B and two to group G (*dna*-399 was incorrectly located by them). They found that F-prime transfer was as efficient at restrictive temperature as at permissive temperature in all cases, indicating either that replication is not required for transfer or that the mode of replication that occurs during transfer does not require the missing *dna* functions. The latter view was supported by the demonstration of a net stimulation of DNA synthesis during mating between all pairs of mutants, and by the recovery of a significant proportion of the DNA labelled during mating between *dnaB* mutants as covalent (F-prime) circles. Quantitative measurements indicated that at least one new F-prime strand was synthesised in each transfer event, although they did not establish whether the newly synthesised DNA ended up in the donor or recipient cells or both.

Transfer of DNA from Hfr to $F^-$ cells probably also involves a type of DNA synthesis that is not dependent on the *dnaB* gene product. Experiments of BONHOEFFER (1966) and BONHOEFFER et al. (1967), in which either donor or recipient cells had a $dnaB^-$ lesion were originally interpreted as indicating a requirement for DNA synthesis in the recipient but *not* in the donor cells. However evidence has since been presented that some kind of restriction of transferred DNA takes place in $dnaB^-$ recipient cells at high temperature (MOODY and LUKIN, 1970), and BRESLER et al. (1968) have observed a stimulation of DNA synthesis, analogous to that found by MARINUS and ADELBERG, induced in $dnaB^-$ Hfr cells by mating (see also CURTISS, 1969).

### 3. Nature of the Functions Affected in *dnaB* and *dnaG* Mutants

Recent work suggests that there is a close parallel between the two modes of replication permitted in *dnaB* (and *dnaG*) mutants, namely DNA transfer in conjugation, and progeny single-stranded phage DNA synthesis in phage M 13-infected cells: each appears to involve continuous displacement of one strand from a "rolling circle" intermediate without concomitant synthesis of a complementary strand (VAPNEK and RUPP, 1970; DRESSLER, 1971). This mode of synthesis is depicted in Fig. 6 which also illustrates schematically the two stages of M 13 replication that do not take place at restrictive temperature in *dnaB* mutants. It will be seen that each of the forbidden modes of replication involves DNA synthesis on single-stranded templates and that one of them, RF replication (Fig. 6b), corresponds to normal semi-conservative replication in models in which one strand is synthesised continuously and the other discontinuously (Fig. 1B and C; p. 40). The function of the *dnaB* and *dnaG* products could therefore be to provide a "priming" mechanism for discontinuous DNA synthesis, or to complex with single stranded DNA to maintain it in an extended configuration. The latter function is analogous to that proposed by ALBERTS (1970) for the gene 32-product of phage T 4. If

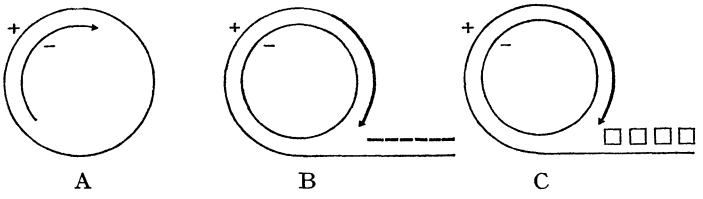


Fig. 6A–C. Probable stages in the replication of phage M13. (A) The infecting single-stranded "+" circle is converted into the parental double-stranded replicative form (SINSHEIMER et al., 1962). (B) Multiplication of replicative form molecules proceeds by a rolling circle mechanism as set out in Fig. 2 (p. 42) (DRESSLER and WOLFSON, 1970). (C) During progeny single-strand DNA synthesis formation of a negative strand complement on the displaced "+" strand is inhibited. In this scheme inhibition is the result of, or involves, complexing of the displaced "+" strand with a phage-specific product which may be the phage M13 gene 5-product (PRATT, 1969; ALBERTS, pers. comm.). An F-coded product, perhaps the pilus itself, may play an analogous role in the case of conjugal DNA transfer

either of these (hypothetical) functions were missing the DNA displaced by continuous synthesis would remain single-stranded and might interfere with further synthesis. In the permitted modes of synthesis on the other hand there must exist a mechanism to *prevent* formation of the complementary strand and this must at the same time permit continued chain elongation on the rolling circles (see Fig. 6, legend). It may be noted that the *dnaB* and *dnaG* products probably perform distinct functions since some of the F-prime DNA synthesised during conjugation was recovered as supercoils in the case of *dnaB* mutants but not in the case of *dnaG* mutants (MARINUS and ADELBERG).

## VII. *dna* Mutants and the Initiation of Chromosome Replication

### 1. Regulation of Initiation

Co-ordination of the rate of DNA synthesis with overall cell growth is achieved in bacteria by varying the interval between successive rounds of replication. Neither the rate of chain elongation nor the length of time between completion of a round of replication and the ensuing cell division vary greatly at different growth rates, at least up to generation times of an hour (COOPER and HELMSTETTER, 1968). A consequence of this is that the average DNA content per cell increases with increasing growth rate. Observations on cultures growing at different growth rates have led to the conclusion that initiation of rounds of replication occurs when the *ratio* of cell mass (or volume) to number of chromosome replication origins attains a certain constant value regardless of growth rate (DONACHIE, 1968).

This type of co-ordination may be understood if initiation is regulated by some product which accumulates in parallel with cell growth (DONACHIE; COOPER and HELMSTETTER; ALBERTS, 1970). (Obviously more than one product could

be involved but for simplicity I shall assume that there is only one.) One can imagine that molecules of this product interact stoichiometrically with the available chromosome origins or with some related sites and that initiation takes place when their concentration attains a certain value relative to the number of target sites, or when they have formed a number of aggregates of critical size. Once initiation has occurred the effective concentration of the product would automatically drop since the number of origins doubles; alternatively the aggregates might be in some way "consumed" in the act of initiation. The regulatory component could equally well be an *inhibitor* which is diluted by growth and interacts stoichiometrically with the chromosomal origin or with other target sites (PRITCHARD et al., 1969). Its concentration would vary in the necessary manner between one initiation and the next if the gene coding for it was situated near the origin of replication and if it was either (1) transcribed for a short time and the product was stable, or (2) transcribed continuously and the product was unstable.

Analysis of *dna* mutants defective in initiation could provide some insight into which type of control is actually employed. In addition it seems probable that other gene products are required in the actual process of initiation; identification of these products could help to distinguish experimentally between the various models of initiation discussed in section I.

## 2. *dna* Mutants Defective in Initiation

I have already mentioned that mutants in groups A and C appear to be unable to initiate rounds of replication at high temperature. It should be emphasised that the strength of the evidence for this varies greatly for different members of these groups and that the grouping itself is tentative. I shall now summarise the available information.

*Residual synthesis.* Much of the evidence that these mutants are defective in initiation rests upon the parallel between their behaviour at high temperature, and the effect of inhibition of protein synthesis, which stops further accumulation, or dilution, of the initiation-controlling product discussed above (MAALØE and KJELDGAARD, 1966). All the mutants make a substantiol amount of DNA after transfer to high temperature, and the amount made agrees reasonably well with that expected if rounds of replication that are underway go to completion. In many cases it has been shown that a similar amount is made at permissive temperature in the presence of chloramphenical, and a few mutants have been shown to carry out no DNA synthesis at high temperature if they are first starved of amino-acids at low temperature until rounds of replication have been completed (*dna*-46A, -47A, -2C). (For sources of information here and throughout see Table.) 1 In two cases (*dna*-46A and -2C) the extent of residual synthesis was observed to depend on the previous rate of growth at low temperature. This is to be expected since the average number of replication forks per chromosome increases with increasing growth rate (OISHI et al., 1964; MASTERS, 1970; CARO and BERG, 1969).

*Resumption of DNA synthesis.* Experiments have been performed in which the DNA synthesised by *dnaA* mutants during recovery from exposure to high temperature was differentially labelled with bromouracil or isotopic precursors. These experiments indicate that re-initiation occurs in a specific region of the chromosome (*dna*-46A) and that this region is the same as that at which synthesis recommences after amino-acid starvation (*dna*-83A). They thus support the idea that the effect of amino-acid starvation as well as of these *dna* lesions is to permit completion of rounds of replication that are in progress but to prevent initiation of new rounds.

*Growth of phage λ.* Phage λ is able to multiply at restrictive temperature in all mutants of groups A and C examined (*dna*-2C, -46A, -252, -325C, and -508A), whereas it fails to multiply in any mutants of the other groups so far tested (see section V). This distinctive behaviour is easily accounted for if lambda codes for its own initiation functions but relies on the host for other functions (JACOB, BRENNER, and CUZIN, 1963).

*Integrative suppression.* Insertion of the F factor or any other plasmid into the bacterial chromosome produces a structure consisting of two replicons in tandem. If the inserted element codes for its own initiation functions insertion could conceivably lead to "rescue" of group A or C mutants at restrictive temperature as a result of passive replication (THOMAS and MOUSSET, 1970) of the chromosome by the inserted element. Several workers (see NISHIMURA et al., 1970) have in fact observed that $F^+$ derivatives of all initiation mutants of group A that have been tested, although still temperature-sensitive, give rise to temperature-resistant "revertants" with increased frequency. There is good evidence that in these "revertants" the F factor has been inserted into the host chromosome to yield a tandem replicon and further that "integrative suppression" results only from certain initiation events, not others. It has not been shown that the origin of replication depends on the site at which F is inserted, nor is it known what determines whether suppression will or will not take place. Other elements that have been shown to bring about suppression in tandem replicons include phage P 1, and an RTF factor (NISHIMURA and CARO, pers. comm.) and possibly certain mutants of phage λ (P. BRACHET, pers. comm.). We have found that integration of an F*lac* factor with a thermosensitive mutation blocking its own replication can lead to integrative suppression (WECHSLER and GROSS, unpublished). The two defective tandem replicons must therefore complement one another in some way in this instance. Integrative suppression has been observed in *dna*-1C but not in *dna*-2C (ZEUTHEN, pers. comm.). The *dna*-2C strain may be a double mutant (B. WOLF, pers. comm.).

*Nature of the initiation defects.* *dna*-1C and -2C and all the *dnaA* mutations tested (*dna*-252A has not been tested) have been shown to be recessive by the use of appropriate F-prime factors (WECHSLER, ZUSSMAN, and GROSS, unpublished). Some indication of the nature of the components affected in these mutants may be obtained by observing the behaviour of the mutants upon return to permissive temperature. Three of the mutants, *dna*-28C, -252A (BEYERSMANN et al., pers. comm.) and -325C, initiate at least one round of

replication rapidly and more or less synchronously upon return to low temperature even if chloramphenicol is present. The products affected in these mutants are therefore reversibly denatured at high temperature, and are probably enzymes involved in triggering replication rather than in controlling the timing of initiation. It is interesting to note that the *colE1* factor is unable to replicate at the non-permissive temperature in *dna*-252A cells (Goebbel, 1970).

Three other mutants, *dna*-83A, -46A, and -47A fail to synthesise any DNA if protein synthesis is prevented upon returning them to low temperature, presumably because the thermosensitive component is irreversibly denatured. If protein synthesis is permitted DNA synthesis resumes after a variable delay; thereafter initiation events occur in more rapid succession than normal and at the end of an hour the rate of replication temporarily exceeds that of control cultures. This last result indicates that the component affected in these mutants is probably not a key protein which accumulates in parallel with growth and regulates the timing of initiation unless it is synthesised at an accellerated rate during the recovery period. The delay that occurs before initiation might be taken as evidence that the affected component is also not an enzyme required in small amounts for initiation. However the delay could be due to damage suffered during incubation at restrictive temperature (see section V).

### 3. Initiation Events Associated with Arrest of DNA Synthesis

Kogoma and Lark (1970) have recently observed that if *E. coli* cells are thymine starved or treated which nalidixic acid for fifty or sixty minutes they not only initiate a new round of replication as soon as DNA synthesis is permitted (see Pritchard and Lark, 1964; Pritchard et al., 1969) but are able to initiate additional rounds, at a reduced rate, for many hours in the absence of further protein synthesis. The nature of this long-lasting aberration of the initiation mechanism is quite unclear but it appears to involve components of the normal initiation mechanism since it is observed at 30° C in *dna*-46A cells and is irreversibly inhibited by transferring them to high temperature (Hirota and Mordoh, quoted by Kogoma and Lark, 1970). In addition it did not occur after exposure of several *dnaB* mutants to high temperature (Kogoma and Lark).

Abnormal initiation of a different kind is induced when the *dnaB* gene product is inactivated. If *dnaB* mutants are exposed to high temperature for a period and then returned to low temperature they initiate a new round of replication as well as continuing the previous round (Stein and Hanawalt, 1969; Inouye, 1969; Kogoma and Lark, 1970; Ricard and Hirota, 1969). At first sight this behaviour appears to be analogous to the immediate effect of thymine starvation already discussed. However studies by Worcel (1970) with *dna*-266B cells suggest that there may be important differences. Exposure to high temperature for only 10 minutes is sufficient to cause re-initiation in all the cells, apparently at a unique chromosomal location and on only one of the two

partially replicated daughter chromosomes. Incubation at high temperature for longer periods does not provoke initiation of additional rounds, but this can be achieved by repeated cycling between high and low temperature, one new fork being induced after each cycle. WORCEL obtained evidence that the choice of a chromosome origin for reinitiation is not random: instead each event involves a unique strand of the parental chromosome. This type of initiation is precisely that expected in the rolling circle model (Fig. 2, p. 42) since, according to that model, a new replication fork can be initiated on the circular component of a partially replicated chromosome, but not on any linear branch. The fact that initiation can occur in this way indicates that there is some intrinsic difference between the two origins of a partially replicated chromosome. Such a difference is not apparent in either of the two most probable models of replication in *E. coli*, namely the "Cairns" model, and the opposing rolling circle model (see section I).

## VIII. Effects of *dna* Lesions on *in vitro* DNA Synthesis

One of the original motives for isolating *dna* mutants was to determine whether any such mutants would be defective in the Kornberg DNA polymerase. The fact that no mutants of this kind were found was a major factor in the evolution of the idea that this enzyme is not essential for DNA replication. With the isolation of *pol*$^-$ mutants and the characterisation of systems of *in vitro* synthesis apparently independent of DNA polymerase I, attention has turned to attempts to demonstrate and analyse defects in DNA replication *in vitro* that are associated with the *dna* mutations.

As I have already mentioned MOSES and RICHARDSON (1970a) found that *dna*-43 B and -266 B cells were defective in DNA synthesis at high temperature in the toluene system they had developed. The same has been found, for a number of *dnaB* mutants, by KOHIYAMA and KOLBER (1970), MORDOH, HIROTA, and JACOB (1970) and PEACEY and GROSS (unpubl.), and BAZILL (pers. comm.) has demonstrated phenotypic reversal (see p. 58) of toluene-treated *dna*-70 B cells by a high concentration of glycerol. In addition MORDOH et al. have shown that if a culture of the initiation-defective mutant *dna*-46 A is incubated at restrictive temperature to allow completion of rounds of replication and then assayed after toluene treatment at high temperature, no synthesis is observed. These results constitute powerful evidence that the synthesis observed in toluene-treated cells corresponds to *in vivo* replication. However they provide no information as to the nature of the defective components. As yet *dna* mutants in the other groups have not been examined in this system but it seems likely that they wil behave as they do *in vivo*, unless they are defective in precursor synthesis.

MOSES and RICHARDSON (1970c) have purified DNA polymerase II from *dna*-43 B and -266 B and have detected no abnormality in the purified enzyme. This result lends support to the evidence, already discussed, that B group mutants are defective in some component other than the "replicase". Of

course direct proof that DNA polymerase II *is* the replicase is still lacking. The inviability of cells carrying a *recA*$^-$ mutation together with the *polA1* mutation, and certain other observations, has suggested to several workers that DNA polymerase II may be the product of the *recA* gene. However the DNA polymerase II activity of a variety of *recA*$^-$ strains appears to be normal (KORNBERG and GEFTER, and MOSES and RICHARDSON, pers. communications; PEACEY and GROSS, unpubl.).

## Conclusion

The evidence I have discussed suggests that *polA1* cells lack Kornberg DNA polymerase activity *in vivo* as well as *in vitro*, and hence that this enzyme is not essential for replication. Experiments with *polA1* cells have led to the discovery of a new DNA polymerising activity capable of carrying out semiconservative synthesis after toluene-treatment of whole cells, as well as in "membrane" preparations obtained by gentle lysis procedures. An enzyme, DNA polymerase II, which is normally membrane-bound, has been purified and shown to differ in a number of ways from the Kornberg enzyme. However the purified enzyme appears unable to do more than fill in gaps in double-stranded DNA and so provides little insight into the mechanism of replication. It seems probable that further progress will depend on analysing other components of the membrane-replication complex.

Evidence for such components comes from two sources. First, analysis of the semiconservative DNA synthesis in toluene-treated cells has revealed a requirement for ATP and $K^+$ ions not shown by purified DNA polymerase II. A number of observations indicate that the ATP may be somehow used to unwind DNA at the growing point (see section III). Second, the examination of a large number of *dna* mutants has demonstrated the existence of five groups of mutants all of which appear to be in some way defective in chain elongation. In the one group of mutants so far examined in any detail, Group B, it has been shown that incorporation of deoxynucleotide triphosphates into DNA is defective at high temperature after toluene treatment, but that DNA polymerase II is apparently normal. The products of the *dnaB* and *dnaG* genes may be required for discontinuous synthesis on single-stranded segments of DNA (see section VI).

There is as yet only circumstantial evidence that DNA polymerase II is the bacterial "replicase". It is important therefore to determine whether it is altered in any of the other *dna* mutants of *E. coli* or *B. subtilis*. A mutant of *B. subtilis* that we isolated some time ago, *mut-1*, is perhaps the best candidate, as it shows very high mutator activity at permissive temperature as well as being thermosensitive for DNA synthesis (GROSS et al., 1968). It is thus similar to many gene 43 mutants of phage T 4.

A number of observations have a bearing on the possible involvement of single-strand interruptions in DNA replication. In discontinuous models of replication synthesis of at least one strand requires joining of polynucleotide

fragments by a ligase. The results of pulse-labelling experiments with a bacterial ligase mutant are consistent with this view, and experiments with *polA1* cells have been interpreted as indicating a requirement for DNA polymerase I as well, presumably because the discontinuities are frequently gaps rather than "nicks". However the possibility remains that selective nuclease action at the growing point may contribute significantly to the observed fragmentation of newly synthesised DNA, and evidence obtained with *dna* mutants indicates that such nuclease action can occur, at least when DNA synthesis is arrested (Section V).

The existence of single strand discontinuities, and of gaps in particular, has been invoked in two other connections: first, to account for the inviability of *polA1 rec*$^-$ double mutants, and second to permit the rotation of DNA that must accompany replication. If single strand gaps are common and play a role in rotation, a mechanism would have to exist to remove them before they reach the replication fork, since chromosome fragmentation would otherwise occur. It is interesting therefore that analysis of repair of radiation damage in *pol*$^-$ and *rec*$^-$ cells suggests that *rec*-dependent repair may provide such a mechanism since it appears to take place in association with the replication fork as it travels along a DNA molecule. The poor growth of *rec*$^-$ cells could be due to inability to repair gaps that reach the replication fork, and the inviability of *rec*$^-$ *pol*$^-$ cells (as well as ligase$^-$ cells) to inability to repair any gaps.

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# Approaches to the Quantitative Analysis of Delayed Hypersensitivity

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## Abbreviations

| | |
|---|---|
| BCG | bacille Calmette Guerin |
| BF | blastogenic factor |
| BGG | bovine gamma globulin |
| BSA | bovine serum albumin |
| DNP-BGG | dinitrophenyl bovine gamma globulin |
| DNP-GPA | dinitrophenyl guinea pig albumin |
| DNP-HGG | dinitrophenyl human gamma globulin |
| GPA-OA | guinea pig albumin orthonilic acid |
| HSA | human serum albumin |
| IDS | inhibitor of DNA synthesis |
| LT | lymphotoxin |
| MAF | macrophage aggregation factor |
| MIF | migration inhibitory factor |
| OT | old tuberculin |
| PHA | phytohemagglutinin |
| Pic HSA | picrylated human serum albumin |
| PPD | purified protein derivative |
| SRBC | sheep red blood cells |

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# I. Introduction

Phenomena as diverse as tuberculin sensitivity, delayed sensitivity to soluble proteins other than tuberculin, contact allergy, homograft rejection, experimental autoallergies, and the response to many microorganisms, have been classified as members of the class of immune reactions known as delayed or cellular hypersensitivity. Similarities in time course, histology, and absence of detectable circulating immunoglobulins characterize these cell-mediated immune reactions *in vivo*. The state of delayed or cellular hypersensitivity can be transferred from one animal to another by means of sensitized living lymphoid cells (CHASE, 1945; LANDSTEINER and CHASE, 1942; MITCHISON, 1954). The responsible cell has been described by GOWANS (1965) as a small lymphocyte. Passive transfer has also been achieved in the human with extracts of sensitized cells (LAWRENCE, 1959).

The *in vivo* characteristic of delayed hypersensitivity from which the class derives its name is the delayed skin reaction. When an antigen is injected intradermally into a previously immunized animal, the typical delayed reaction begins to appear after 4 hours, reaches a peak at 24 hours, and fades after 48 hours. It is grossly characterized by induration, erythyma, and occasionally necrosis. The histology of the delayed reaction has been studied by numerous investigators (COHEN et al., 1967; GELL and HINDE, 1951; KOSUNEN, 1966; KOSUNEN et al., 1963; MCCLUSKEY et al., 1963; WAKSMAN, 1960; WAKSMAN, 1962). Initially dilatation of the capillaries with exudation of fluid and cells occurs. At 24 hours, the large proportion of these cells are mononuclear leucocytes, both lymphocytes and macrophages. Studies with $H^3$ thymidine have established that the majority of the cells are from a hematogeneous proliferating pool (KOSUNEN et al., 1963). Passive transfer work has established that less than 10% of the cells are specifically sensitized, and that the remainder are non-specific cells (COHEN et al., 1967; MCCLUSKEY et al., 1963). The latter have been shown by LUBAROFF and WAKSMAN (1967, 1968a, 1968b), with the use of a fluorescent labelling technique, to originate in the bone marrow. Elucidation of the role of the sensitized cells and identification of the factors which cause the appearance of non-specific cells at the site of inflammation are closely related problems. Their solution would provide a basis for understanding the mechanism of delayed hypersensitivity.

Tissue damage is a prominent feature of delayed reactions. Characteristic lesions can be described as:

(1) the invasive-destructive lesion: infiltrating histiocytes appear to exert a direct destructive effect upon parenchymal elements which contain antigen; (2) the vasculonecrotic lesion: vessels are impregnated with fibrinoid and polymorphonuclear leukocyte exudation occurs; (3) massive necrosis: seen in the center of strong tuberculin reactions (WAKSMAN, 1962). Such destructive reactions have been seen in the tuberculin reaction (GELL and HINDE, 1951), allergic encephalomyelitis (PATERSON, 1962), thyroiditis (ROITT et al., 1962; ROSE et al., 1962), contact dermatitis (FLAX and CAULFIELD, 1961), tumour

homografts (KOSUNEN et al., 1963), and graft versus host reactions (ELKINS, 1964, 1966).

Proliferation has also been associated with delayed hypersensitivity. GELL and HINDE (1951) described proliferation of the epidermis in the skin test site of a positive tuberculin reaction.

Fever is a systemic manifestation of delayed hypersensitivity, which is produced by an intravenous injection of antigen into a sensitized animal. The fever is often accompanied by lymphopenia. This subject has been reviewed by ATKINS (1960, 1965).

Macrophages "disappear" from the peritoneal exudates of guinea pigs with delayed hypersensitivity after antigen injection. This loss is not due to cell death but to an increased adherence of the cells to the omentum. The reaction cannot be transferred with serum, although the influence of a cytophilic antibody has not been eliminated. Smaller numbers of macrophages are seen when antigen is injected with cells treated with serum from sensitive donors (NELSON, 1963, 1966; NELSON and BOYDEN, 1963; NELSON and NORTH, 1965).

Though many *in vivo* manifestations have been associated with delayed hypersensitivity, none can be measured with the precision with which one is able to quantitate antibody production. Due to the absence of a mediator in the circulation in large quantities, quantitative tissue culture approaches to delayed hypersensitivity are useful. The complexity of the delayed reaction is another reason *in vitro* approaches should be optimized. The duration of graft survival and skin test diameters do not give an indication of the activity of sensitized cells alone, since both specific and non-specific cells are involved. One cannot think in terms of mechanism, kinetics, and theories of immune response and its evolution until one can consider the delayed response in quantitative terms. Clinical advances can be made in diagnosing autoallergies and graft rejection and in facilitating their treatment only if the systems can be evaluated in an isolated environment.

Numerous criteria can be established for the ideal *in vitro* method of analyzing cellular hypersensitivity. In point of fact, all such criteria are arbitrary; some are less important than others, and can be sacrificed if the situation demands. Many of the criteria should be applied to all *in vitro* methods, and some are unique to cellular hypersensitivity.

One essential criterion is a correlation of the *in vitro* method with *in vivo* characteristics of cellular hypersensitivity, such as the delayed skin reaction and graft rejection. Lymphoid cells from an animal expressing the delayed response should perform optimally in the method under investigation. Those from an animal sensitized in such a way as to make antibody alone should be ineffective or at least, less effective. Carrier specificity should be present. It should be possible to do passive transfer of sensitized lymphoid cells from one animal to another and concurrently transfer the ability to perform the *in vitro* activity. The method should not work well with cells from individuals with impaired cellular hypersensitivity and immune deficiency states such as

Hodgkin's disease and sarcoidosis. Cells which are not immunocompetent, such as bone marrow and thymus, should be unable to function alone *in vitro*. Cells which are functioning *in vitro* should be performing a task which they do in the animal. The system should be reasonably physiologic with a time course, antigen concentration and cell number comparable to that observed *in vivo*. If the system is relevant to the *in vivo* situation, a certain flexibility and unity should exist in that it should be applicable to cases of delayed hypersensitivity to soluble antigens, autoallergies, viruses, and homografts.

The results of a good test for delayed hypersensitivity should be expressed in a quantitative, non-subjective manner. Quantitative interpretations of delayed reactions *in vivo* are usually based on the diameter of skin test reactions, the amount of induration (expressed in a scale of 0 to ++++), clinical symptoms in an autoimmune disease, and mean survival time of skin grafts (measured in days). None of these express the activity of sensitized cells alone and all are influenced by the activity of non-sensitized cells to some extent. Interpretation of the number obtained *in vitro* is usually difficult, but should be an expression which can be related to the activity of individual cells.

The component parts of the reaction should be separable and clearly distinguishable to permit reasonable quantitative interpretations. For example, in a cytotoxic system, the effector lymphocyte activity should be distinguishable from the target activity. One should be able to perform the activity *in vitro* with those cells characteristic of the *in vivo* situation. Ideally one should be able to separate sensitized cells from non-sensitized cells.

Other desiderata of the ideal quantitative approach include technical ease, reproducibility, low variation, low background, negative controls, small numbers of lymphocytes, provision of kinetic information, relative speed, performance with standard equipment, and applicability to cells of the animal species to be studied. One should be able to relate the number obtained to the individual cell. The latter is an ideal which cannot be realized in any of the systems except those which permit an evaluation of individual cells fairly early in their interaction with antigen, before recruitment and cell division have occurred.

This paper will include a brief description of the methodology of most of the *in vitro* methods used to quantitate delayed sensitivity, tables which contain references to key uses of a particular method, and an evaluation of each in terms of its fulfilling the criteria established above. Methods for evaluating homografts, delayed sensitivity to soluble antigens and viruses, and autoallergies will be included. The mixed lymphocyte reaction and other methods for assessing histocompatibility which could be considered a primary response, assays of graft vs. host disease, and tumour immunity will not be treated in detail. Experiments with phytomitogens (phytohemagglutinin, pokeweed mitogen, and conconavallin A) will not be emphasized. The non-specific nature of the substances militates against their consideration as immunologic agents. Work with lymphoid derived cell lines (GLADE and HIRSCH-

HORN, 1970) will also not be discussed; though the products of these cells excreted into the medium appear to resemble MIF and LT in their effects, the system is not one which measures an immune response to a particular antigen.

The reader who is interested in further study of the topics discussed in this paper should be aware of other reviews which have appeared recently. These include an excellent review of tumour immunity (HELLSTRÖM and HELLSTRÖM, 1969), the World Health Organization Symposium (1969), a review by PERLMANN and HOLM (1969), and the results of 2 symposia, one edited by LAWRENCE and LANDY (1969) and the other by BLOOM and GLADE (1971).

The phenomena which will be described here result from the interaction of antigen with specifically sensitized lymphocytes. This interaction results in a change in the lymphocyte itself which can be ascertained morphologically or chemically, in the production of a substance, or in an effect on a target which reacts with the lymphocyte or with its product. The systems will be evaluated in terms of the criteria and desiderata mentioned above.

## II. Blast Cell Transformation

Lymphocytes undergo a dramatic transformation when they are confronted with an antigen to which they have been sensitized. A small number of cells in the antigen-exposed population undergo changes which cause them to resemble a more primitive form called a lymphoblast. Increased basophilia, the appearance of nucleoli, diffuse chromatin, increased staining of lysosomal enzymes are all obvious and distinctive changes noted in the cultures. Eventually, an increase in the number of mitotic figures also occurs. These changes can be evaluated in terms of increased DNA synthesis, qualitative and quantitative changes in RNA synthesis (COOPER and RUBIN, 1965), and increased protein synthesis. Phytohemagglutinin and other nonspecific agents induce blast transformation in cultures of normal lymphocytes and affect a greater proportion of cells than other methods of stimulation. Three reviews of blast transformation have been published (ROBBINS, 1964; OPPENHEIM, 1968; LING, 1968), and the system has been used so extensively that it will be covered only briefly here. The book by LING (1968), is particularly helpful and provides an excellent bibliography. The methodology of blast transformation is technically simple. Lymphoid cells from spleen, lymph node, peripheral blood, or thoracic duct are most commonly incubated at concentrations of approximately $1 \times 10^6$ cells/ml in 2 ml of medium in capped culture tubes in the presence of approximately 20 micrograms antigen/ml, in a moist atmosphere of 95 % air, 5 % $CO_2$. Generally after 72 hours, the cultures are harvested and blast transformation assessed by various techniques. These include staining for morphologic observation, uptake of radioactive nucleic acid precursors such as $H^3$thymidine, $H^3$uridine, $C^{14}$thymidine, or $C^{14}$leucine (WEINER et al., 1969).

Table 1. *Blast cell transformation*

| Author | Source of cells | Antigen | Measurement |
|---|---|---|---|
| PEARMAIN et al. (1963) | human, peripheral blood | OT | mitotic frequency |
| ZWEIMAN et al. (1966) | guinea pig, peripheral blood | OT | mitotic fequency |
| MILLS (1966) | guinea pig, lymph node | DNP-BGG | morphology, $H^3T$ incorporation |
| OPPENHEIM et al. (1967) | guinea pig, lymph node, spleen, thymus and peripheral blood | PPD GPA-OA | $H^3T$ incorporation |
| DUMONDE et al. (1969) | guinea pig, lymph node | BGG | 1) $H^3T$ incorporation 2) supernatant causes increased $H^3T$ incorporation in normal lymph node cells |
| MAINI et al. (1969) | human, peripheral blood | PPD | 1) $H^3T$ incorporation 2) supernatant causes increased $H^3T$ incorporation in normal peripheral blood lymphocytes |
| DAU and PETERSON (1969) | rat, spleen | human brainstem | $H^3T$ incorporation |
| WOLSTENCROFT and DUMONDE (1970) | guinea pig, lymph node | BGG PPD GPA-OA | 1) $H^3T$ incorporation 2) supernatant causes increased $H^3T$ incorporation into normal lymph node cells |
| ADLER et al. (1970) | mouse, spleen | PHA PPD | $H^3T$ incorporation |

Supernatant from cultures undergoing blast transformation can be added to normal lymphocytes and cause increased DNA synthesis (DUMONDE et al., 1969; MAINI et al., 1969). The substance is called "blastogenic factor", and is probably the same as that described by VALENTINE and LAWRENCE (1969) which appears under similar conditions and which causes normal lymphocytes to undergo blast cell transformation only in the presence of specific antigen.

The relationship of blast transformation to delayed hypersensitivity has been established. Carrier specificity exists (MILLS, 1966; OPPENHEIM et al., 1967), and it has been claimed that lymphoid cells from animals immunized intravenously in such a way to produce antibody do not undergo blast trans-

formation. However, spleen cells from immunized mice which are making antibody also respond by proliferation when confronted with specific antigen *in vitro* (DUTTON, 1967), and blast transformation can be induced by antigen-antibody complexes (MÖLLER, 1969). A temporal relationship exists between the time of appearance of the ability to transfer delayed hypersensitivity with the appearance of blast reactivity in cultures (OPPENHEIM et al., 1967). Another correlation with delayed hypersensitivity is the concommitant impairment of a patient's lymphocytes' ability to undergo blast transformation in diseases associated with impaired cellular hypersensitivity, such as Hodgkin's disease, sarcoidosis, and thymic aplasia (OPPENHEIM, 1968).

Quantitation of blast transformation is technically simple, but difficult to interpret and relate to individual responding cells. For example, DNA synthesis as measured by an increase in tritiated thymidine uptake could reflect the activity of a small number of cells that are synthesizing a great deal of DNA, many cells synthesizing slightly elevated amounts of DNA, or a combination of the two possibilities. Methods that attempt to probe the single cell, such as measurements of increased cell size, are tedious and subjective. Blastogenic factor makes interpretation more difficult, since it can recruit normal lymphocytes into the system by stimulating DNA synthesis. Kinetic studies should probably be performed more frequently in cases of blast transformation, since one is interested in the rate and total amount of DNA synthesis, and certain populations may reach peak synthesis that is higher but at a different time. The exciting prospect of unravelling early events at the single cell level is within the capability of the system.

The technical ease and small numbers of cells that blast cell transformation requires make it a popular method, especially for use with humans. Other species have been more difficult, though the guinea pig has been used. Recent work with mouse lymphocytes (ADLER et al., 1970) is quite promising.

Table 1 summarizes cells, antigens and measurements used in a number of studies on blast cell transformation.

## III. Inhibition of Macrophage Migration

Early studies of tuberculin sensitivity *in vitro* were concerned with the cytotoxic and inhibitory effects of antigen on mixed populations of cells from sensitized animals and were somewhat subjective. HOLST (1922) found that tuberculin was more toxic for white blood cells from a tuberculous animal than from a normal healthy one. RICH and LEWIS (1928, 1932) confirmed these findings and found that tuberculoprotein also was toxic to and inhibited the migration of cells from explants of spleen and buffy coat. The original experiments with tuberculin were confirmed and extended with many other antigens, and have been reviewed by HEILMAN (1963). See also ŠVECJAR and JOHANOVSKY (1961a, 1961b, 1961c, 1965) and ŠVECJAR et al. (1965).

Recent studies of the migration inhibition system have been more quantitative. GEORGE and VAUGHAN (1962) devised the capillary tube procedure which has been used by DAVID et al. (1964a) and many others. Peritoneal exudate cells from a sensitized animal are placed in a capillary tube and packed, the tube is cut and placed in a chamber which contains antigen and medium. Migration of the cells from the tubes is observed at 24 hours. Migration of sensitized cells into the medium is inhibited in the presence of specific antigen. The area of migration is projected onto a screen and measured by planimetry. The absolute area of migration is calculated in reference to a projected standardized square millimeter.

The migration index is calculated as:

$$\% \text{ migration} = \frac{\text{average migration with antigen}}{\text{average migration without antigen}} \times 100 .$$

Alternatively, the image of the area of migration can be drawn on transparent paper which is then cut and weighed. The weight of the paper is considered the area of migration (FALK et al., 1970).

The mechanism of migration inhibition is fairly well understood. DAVID et al. (1964b) demonstrated that a small number of sensitized cells inhibited a larger number of normal cells in the presence of specific antigen. Sensitized lymphocytes release a factor into the medium 8 hours after interaction with antigen. The factor called MIF (migration inhibitory factor) inhibits the migration of normal macrophages (DAVID, 1966; BLOOM and BENNETT, 1966).

The phenomenon of migration inhibition correlates well with delayed hypersensitivity (ARONSON, 1931, 1933; HALL and SCHERAGO, 1957a, 1957b; HEILMAN et al., 1944; HEILMAN and FELDMAN, 1946; HEILMAN and SEIBERT, 1946; MOEN, 1936, 1937). It is generally difficult to produce with cells from animals with circulating antibody. The *in vitro* manifestation correlates in time with *in vivo* delayed skin reactions (DAVID et al., 1964b). It is hapten specific (DAVID et al., 1964c). DAVID and SCHLOSSMAN (1968) demonstrated that the antigen requirements *in vitro* were the same as those of the *in vivo* delayed and anamnestic responses. Thus a heptamer of 7 amino acids or larger of $\alpha$ DNP (lysine) is required to elicit MIF from guinea pigs sensitized with $\alpha$ DNP $(\text{lysine})_{18}$ peptides. One exception to the correlation of migration inhibition with delayed hypersensitivity is the inhibition of the migration of normal macrophages by antigen-antibody complexes (AMOS and LACHMANN, 1970).

The technique of inhibition of macrophage migration has been used in the study of cellular hypersensitivity using antigens other than soluble proteins. These include: autoallergic encephalomyelitis (DAVID and PATERSON, 1965; BROCKMAN et al., 1968), immunity to fibroma virus (TOMPKINS et al., 1970), and homograft sensitivity. Cells from the graft donor can be used as both antigen and indicator cell (AL-ASKARI et al., 1965) or sonicated cells from the graft donor can serve as antigen *in vitro* (FALK et al., 1970).

A theoretical correlation of the migration inhibition system with the *in vivo* situation exists. The cells involved *in vitro* are those that participate *in vivo* in the delayed skin reaction and in graft rejection. The proportion of sensitized to non-sensitized cells is comparable. Sensitized cells at the skin test site could react with antigen and release a substance which would cause the accumulation of non-specific marrow derived mononuclear cells. Substances which contain MIF when injected into the skin of normal animals cause a lesion which histologically resembles a delayed skin reaction (BENNETT and BLOOM, 1968; HEISE and WEISER, 1969; PICK et al., 1969). However, there are definitely also other factors present in these substances. For example, a chemotactic factor that attracts mononuclear cells is produced under the same conditions and concurrent with MIF. The two substances are separate entities as shown by gel electrophoresis (WARD and DAVID, In: LAWRENCE and LANDY, eds. 1969, p. 262–266; WARD et al., 1970). Production of a purified antibody to MIF which caused prolongation of graft rejection or abolition of a delayed reaction *in vivo* and also inhibited the *in vitro* system would be supportive evidence for the authenticity of the system. Experiments in this area have not ruled out the possibility that the antibody is not antilymphocyte globulin or directed against some other lymphocyte product (LAWRENCE and LANDY, eds. 1969, pp. 296, 345). A theoretical correlation between the mechanism responsible for the macrophage disappearance mentioned above and the migration inhibition system can be suggested on the basis that both appear to involve "stickiness" of the macrophage.

There are some disadvantages to the migration inhibition system. Extrapolation of percent migration to the number of sensitized cells in a population is difficult. Though technical difficulties have been described, they are not insurmountable. Aggregation of macrophages in a test tube can be observed macroscopically (LOLEKHA et al., 1970) and this aggregation, caused by MAF (macrophage aggregation factor), can be graded on a + to ++++ scale. The lack of precise quantitation is believed to be compensated for by the ease of the test.

The migration inhibition system has been used rather infrequently in animal species other than the guinea pig, though it has been used in the mouse, rat and rabbit (FRIEDMAN et al., 1969 and see Table 2). The relatively infrequent use may be explained by species differences in migratory ability of macrophages. Early work with human cells was hampered by the absence of readily available peritoneal exudate cells, though THOR and DRAY (1968a) were able to use lymph node cells as targets. MIF from human cells inhibits the migration of guinea pig peritoneal exudate cells (THOR et al., 1968). BENDIXEN (1967) and SØBERG and BENDIXEN (1967) reported successful migration with human peripheral blood cells as the source of both effector cells and target, as did FALK et al. (1970). These reports were contradicted by KALTREIDER et al. (1969) who reported very poor results with human buffy coat cells and no correlation between migration inhibition and skin sensitivity. Recent work by ROSENBERG and DAVID (1970) now suggests that the MIF system, if

Table 2. *Migration inhibition*

| Author | Effector | Soluble antigens | Target |
|---|---|---|---|
| DAVID (1965, 1966); | guinea pig, lymph node or peritoneal exudate cells | PPD<br>Ovalbumin | guinea pig, peritoneal |
| DAVID et al. (1964a–d); | MIF | diphteria toxoid<br>DNP-GPA | exudate cells |
| DAVID and SCHLOSSMAN (1968) | | O-chlorobenzoyl bovine gamma globulin; DNP $(Lys)_{18}$ peptides | |
| BLOOM and BENNETT (1966) | guinea pig, peritoneal exudate, lymphocytes from sensitized animals MIF | PPD | guinea pig, normal peritoneal exudate macrophages |
| THOR (1967) | human, lymph node cells | PPD<br>histoplasmin | human, lymph node cells |
| THOR et al. (1968) | human, peripheral blood lymphocytes, MIF | histoplasmin<br>PPD<br>coccidiodin | guinea pig, normal peritoneal exudate cells |
| LOLEKHA et al. (1970) | guinea pig, peritoneal exudate cells, lymph node cells, spleen cells, peripheral blood white cells, (MAF) | egg albumin<br>PPD | guinea pig<br>normal peritoneal exudate cells |
| ROSENBERG and DAVID (1970) | human, peripheral blood | PPD | human, peripheral blood |
| TOMPKINS et al. (1970) | rabbit, peritoneal exudate cells | *viral antigens*<br>shope fibroma virus (in infected mono-layers) | peritoneal exudate cells |

| | | | |
|---|---|---|---|
| David and Paterson (1965) | guinea pig, peritoneal exudate cells from animals with allergic encephalomyelitis | *autoallergies* adult rat, nervous tissue | guinea pig, peritoneal exudate cells |
| Brockman et al. (1968) | guinea pig, lewis rat, peritoneal exudate cells from animasl with allergic encephalomyelitis | bovine spinal cord | guinea pig, rat, peritoneal exudate cells |
| Al-Askari et al. (1966) | mouse, peritoneal exudate cells (CBA) | *transplantation antigens* *immunizing* A/Jax skin graft | peritoneal exudate cells (A/Jax) |
| | | *in culture tube* peritoneal exudate cells (A/Jax) | |
| Falk et al. (1970) | mouse, rat, human peripheral blood | skin grafts cell injection | spleen cells, peripheral blood lymphocytes |
| | | sonicated cells | |

performed as outlined by them, can be used with cells from human peripheral blood.

Advantages of the migration inhibition system are considerable. The system is reproducible with statistically significant results. It is also fairly inexpensive in terms of equipment and the small numbers of cells needed.

Migration inhibitory factor (MIF) has probably been the most intensively studies of the various factors in delayed hypersensitivity. It is a protein of molecular weight 35,000–55,000 (Remold et al., 1970). More precise characterization has been hampered by problems of producing it in quantities sufficient for biochemical analysis. Work with concanavallin A and continuous lymphocyte lines may allow the production of larger quantities.

## IV. Macrophage Activation

Sensitized lymphoid cells in the presence of specific antigen cause macrophages to become "activated". The activation has been described as various alterations in appearance or function, all of which are probably comparable. They include an increase in cell size and neutral red uptake (Waksman and Matoltsy, 1958; Švejcar and Johanovský, 1961a), increased adherence to glass or plastic, amoeboid activity, and increased lysosomal enzyme staining. Soluble factors which are released into the supernatant after lymphocyte antigen interaction cause these changes (Mooney and Waksman, 1970; Adams et al., 1970). The relationship of increased adherence and

amoeboid activity to the inhibition of migration described in Section II has not been defined. The phenomena may be manifestations of identical changes in macrophages.

The phenomenon of macrophage activation can be applied to *in vivo* events, though it has not been correlated with delayed hypersensitivity in each case. MACKANESS (1969a, 1969b) demonstrated that spleen cells from mice infected with BCG were activated in their ability to destroy the infecting organism and other unrelated organisms. Increased activity in clearing *Listeria monocytogenes* could be passively transferred to normal mice with living spleen cells from mice sensitized to that organism. BARNET et al. (1968) described a surprising specificity in macrophage activity which is inconsistent with the work of MACKANESS (1969a, 1969b). When lymphoid cells from rabbits sensitized to sheep red blood cells were incubated in the presence of specific antigen, non-specific antigen (rooster red blood cells), and normal macrophages, the latter were stimulated to engulf only the sheep red blood cells. One is tempted to think in terms of opsonizing antibody in this case. The author's statement that serum from these animals did not cause increased phagocytosis does not completely exclude the possibility.

The system of macrophage activation has great potential. Though quantitation is difficult presently, increases in enzyme activity and respiration are amenable to analysis with more sophisticated techniques. Though the assay has not been used to evaluate delayed hypersensitivity in humans, this should be possible has peritoneal exudate cells from other species could be used as targets, as in the MIF system (THOR et al., 1968).

## V. Macrophage Spreading Inhibition

FAUVE and DEKARIS (1968) and DEKARIS et al. (1969) have described a phenomenon in which peritoneal exudate cells from sensitized mice or guinea

Table 3. *Macro-*

| Author | Effector cells | Antigen |
|---|---|---|
| WAKSMAN and MATOLTSY (1958) | guinea pig, peritoneal exudate | OT or PPD |
| BARNET et al. (1968) | rabbit, lymph node | SRBC |
| MACKANESS (1969) | mouse, spleen | BCG |
| MOONEY and WAKSMAN (1970) | rabbit, lymph node | PPD<br>HSA |
| ADAMS et al. (1970) | guinea pig, peritoneal exudate | PPD |

pigs were *inhibited* from spreading in the presence of specific antigen. This phenomenon is observed microscopically and must be read within 1 hour after mixing sensitized cells and antigen. An apparent contradiction with the system of macrophage activation described above in which spreading was *increased* in the presence of specific antigen can be resolved by comparing the different time courses. The spreading inhibition occurs early (1 hour) and the increased adherence occurs after 24 hours. The FAUVE system has not been quantitated and is tedious technically, but a correlation with the delayed skin reaction has been observed.

## VI. Interferon

Interferon is released into the supernatants of cultures of sensitized lymphocytes after antigen exposure. GLASGOW (1966) added Chikungunya virus to cultures of peritoneal exudate cells from mice sensitized to that virus. A protective effect of the supernatant fluid against the cytopathogenic effect of virus was demonstrated in other cultures. WHEELOCK (1965) observed a protective effect against Sindbis virus of supernatants from human leukocytes which had been treated with phytohemagglutinin. Supernatants from cultures of human peripheral blood exposed to antigen to which the donors were sensitive, such as PPD, tetanus toxoid and diphteria toxoid, provided a protective effect against polio, vesicular stomatitis virus, sindbis, and vaccinia (GREEN et al., 1969). Quantitation in this system depends on the protective effect of interferon in terms of percent plaque reduction or dilution end point. The system is somewhat cumbersome, since one has to contend with one more variable (the virus). The relationship of this system to cytotoxic systems (Section VII) is unclear. Interferon which prevents a cytotoxic effect in tissue culture cells is produced under the same conditions as lymphotoxin

*phage activation*

| Target | Observation | Soluble mediator |
|---|---|---|
| same as effector cells | increase in cell size and uptake of neutral red | — |
| peritoneal exudate cells from normal rabbit | stimulation of phagocytosis of SRBC | — |
| peritoneal exudate cells from normal mice | decreased survival of *Listeria monocytogenes* increased spreading on glass | — |
| peritoneal exudate cells from normal rabbit | increased adherence, amoeboid activity | yes |
| peritoneal exudate cells | increased size, adherence, lysosomes and acid phosphatase | yes |

(LT) which causes a cytotoxic effect. The system is a physiologic one since it parallels an activity which occurs in the organism. Experiments to establish an exact correlation with delayed hypersensitivity have not been performed.

Table 4. *Interferon*

| Author | Effector cells | Antigen | Virus inhibited |
|---|---|---|---|
| WHEELOCK (1965) | human, peripheral blood | PHA | Sindbis |
| GLASGOW (1966) | mouse, peritoneal leukocytes or macrophages | Chikungunya virus | Chikungunya |
| GREEN et al. (1969) | human, peripheral blood | tetanus toxoid<br>PPD<br>diphteria toxoid<br>PHA | polio, vesicular stomatitis, sindbis, vaccinia |

## VII. Cytotoxicity

Cytotoxic and inhibitory methods have been extensively used for *in vitro* determinations of delayed hypersensitivity. Their early popularity stems from their technical ease and their relationship to the *in vivo* situation in which tissue damage and cell death are frequent concommitants of delayed hypersensitivity. However, some serious drawbacks have become apparent and they will be discussed in the following description of individual methods.

### 1. Gross Observation

Gross observation of cell death was the earliest assessment of cytotoxicity. GOVAERTS (1960) observed that lymphocytes from a dog which had received a kidney graft would destroy donor kidney cells in culture. He observed "cytotoxic lesions" and stained for viability with trypan blue and eosin. This work was confirmed by MERRILL et al. (1960) with rabbit homografts and ROSENAU and MOON with mice. ROSENAU and MOON (1961, 1962a, 1962b, 1963) using inbred mice showed that the lymph node cells from BALB/C mice immunized with $C_3H$ skin grafts destroyed L cells (of $C_3H$ origin) in tissue culture. They analyzed the mechanism of this cytotoxic effect by attempting to prevent it with chemical inhibitors such as hydrocortisone (1962), and observed the effect using time-lapse cinematography. However, there was no quantitative aspect to this work, either in the effect on the target or the number of cells necessary. Attempts were made to correlate the system with delayed hypersensitivity and not antibody formation; the system worked without the addition of exogeneous complement and the effect was not mediated by serum. BRONDZ (1964) used a similar system with sarcoma and

macrophage monolayers which were destroyed by lymphocytes from mice sensitized with sarcoma cells which differed at the H-2 locus. Lymphocytes from animals with autoallergic diseases (either of suspected autoimmune etiology or induced experimentally) kill targets which contain antigens to which they are sensitized. These phenomena are outlined in Table 5. KOPROWSKI and FERNANDES (1962) showed that Lewis rats injected with guinea pig nervous tissue exhibited experimental allergic encephalomyelitis. Their lymph node cells destroyed glial cells from puppy brain in culture. The advantage of these early studies lay in their technical ease, the disadvantage in their subjectivity.

## 2. Plaques

Plaquing in a monolayer is a somewhat more quantitative approach to observing cytotoxity. One plates a target monolayer, allows it to grow and then adds lymphocytes sensitized against the antigen expressed by the monolayer. After 24–48 hours the monolayer is washed, fixed and stained. Plaques or areas of clearing which are apparent where the monolayer has been destroyed are judged on the basis of 0 to ++++. This is a gross method, but it does enable one to titrate the number of cells needed to produce a plaque, can be assessed rapidly, and can include a number of titrations on the same plate. The plaque can be the result of both cytotoxicity and inhibition of proliferation if the monolayer is not confluent at the time that the lymph node cells are added. The method is useful for rapid screening. It also allows the use of early primary cultures which is a particular advantage in humans especially for clinical applications. For example, kidney rejection could possibly be determined by adding recipient lymphocytes to donor fibroblasts. LUNDGREN et al. (1970) have not been successful with this approach in detecting kidney graft rejection in humans probably due to immunosuppressive treatment of the recipient. The method is effective as an indication of skin graft rejection in untreated individuals and is sensitive enough to discriminate between 1 antigen and 4 antigen differences. Sensitivity to *Salmonella* bacteria in humans can also be detected in this way (LUNDGREN et al., 1970). Lymphocytes from individuals with *Salmonella* infections kill target monolayers cells in the presence of specific antigen.

## 3. Cell Counts

Cytotoxic methods for determining delayed hypersensitivity became more quantitative in the work of WILSON (1963, 1965a, 1965b). Lymphoid cells from animals sensitized with homografts kill kidney or tumour cells of donor origin *in vitro* in approximately 48 hours. Target cell death can be determined by counting in a haemocytometer (WILSON, 1963), or with an electronic particle counter (WILSON, 1965a, 1965b). By means of this method WILSON could easily distinguish the lymphocytes and the target cells on the basis of volume differences. A relationship to delayed hypersensitivity was suggested

by the fact that isoimmune sera did not enhance the cytotoxic effect of sensitized cells (WILSON, 1965a; MÖLLER, 1965a). This relationship was also suggested by the time of appearance of cytotoxic cells in the lymphoid organs, and the absence of a complement requirement (WILSON, 1965a).

The cytotoxic system with the coulter counter as a method of quantitation was also used by RUDDLE and WAKSMAN (1967, 1968a, 1968b, 1968c) in a rat model using soluble antigens. Lymphocytes from an animal sensitized to egg albumin or tubercle bacilli kill fibroblasts of embryonic origin in culture only in the presence of specific antigen. The system is effective with Lewis lymphocytes and Lewis fibroblasts and also with allogeneic target cells. Target cell origin is immaterial. Immunologic specificity resides in the early interaction of antigen with sensitized lymphocyte which results in a toxic effect on an innocent bystander cell. The system is similar in concept to that described above in which MIF inhibits normal macrophages of syngeneic, allogeneic or xenogeneic origin (DAVID et al., 1964a; AL-ASKARI et al., 1966; THOR et al., 1968). RUDDLE and WAKSMAN (1968b) also determined the relationship of the system to delayed hypersensitivity employing criteria already applied in other systems: correlation with appearance of delayed skin reactions *in vivo*, carrier specificity, time course and apparent lack of a complement requirement. A cytotoxic factor was described in the supernatant (RUDDLE and WAKSMAN, 1968c).

The cytotoxic system can be applied to viral immunity (SPEEL et al., 1968) and autoallergic disease (ELLISON et al., 1970). Lewis and Sprague Dawley rats were injected with spinal cord in adjuvant which resulted in experimental allergic encephalomyelitis. Lymph node cells from animals sensitized in this manner killed syngeneic or allogeneic fibroblasts in the presence of basic protein contained in a spinal cord extract.

Certain objections have been raised to the method of counting surviving cells (PERLMANN and HOLM, 1969). One difficulty inherent in the system as described by RUDDLE and WAKSMAN (1967), is the preparation of embryonic fibroblasts. Primary cell lines may give uneven growth characteristics, and are more difficult to prepare. Primary fibroblasts in later passages appear more resistant to the cytotoxic effect of sensitized cells (ELLISON et al., 1970). Embryonic lines were used in the original work because at that time it was not known whether allogenic inhibition (MÖLLER, E. 1965b) would affect the results. Since it did not, it became apparent that almost any cells could be used as targets. Many established cell lines have been used in the system (WILLIAMS and GRANGER, 1968, 1969; LEIBOWITZ and LAWRENCE, 1969). The system is reproducible with little variation among cultures and there is rarely any background death. Purified lymphocytes have been used as effectors in cytotoxic systems, thus invalidating the argument that polymorphonuclear leukocytes might be causing some cytotoxicity. This is unreasonable in any event since controls clearly demonstrate that sensitized lymph node cells in the absence of antigen are not cytotoxic. The objection (PERLMANN and HOLM, 1969) that cells may detach non-specifically from the glass does not

seem a valid one since one is looking for a difference between experimental groups and controls in measuring delayed hypersensitivity, and this detachment should be the same in each group. As long as controls and duplicates are provided there should be no problem. One disadvantage of a system that employs the counting of target cell survival 48 hours after addition of lymphocytes is that it is a dynamic one. Confusion may arise since one is measuring simultaneously cell death, inhibition of proliferation, and proliferation. However, this disadvantage can be obviated by using irradiated target cells (WILSON, 1963). Cytotoxic systems can provide kinetic information which the system employing isotope release cannot (see section F).

One serious disadvantage of the cytotoxic system is the fairly large number of lymphocytes that must be used ($1.5–2.0 \times 10^7$) which is much higher than that needed in either migration inhibition or blast transformation and even in some of the other cytotoxic assays. Micromethods have been described (ULRICH and KIELER, 1969; TAKASUGI and KLEIN, 1969), but they suffer from being tedious and more subject to the vagaries of concentration differences.

### 4. Inhibition of Cell Function

Inhibition of target cell function is a more sensitive indicator than a gross assessment of death. One interesting system was described by FRIEDMAN (1964). Spleen cells from $C_3H$ mice immunized against A-strain mice could prevent spleen cells from A mice sensitized to sheep red blood cells from making plaques in lawns of sheep red blood cells under agar. The assay can be accomplished fairly rapidly after 1 or 2 hours incubation of cells from $C_3H$ and A mice. Another example of antigen-lymphocyte interaction which results in a diminished function of target cells, is decreased uptake of labelled amino acids into tissue culture cells. GRANGER and WILLIAMS (1968) described this method and used it in 1969 as an assay for lymphotoxin. Lymphotoxin is released from lymphoid cells from tuberculin sensitized mice, guinea pigs, or humans after incubation with PPD and causes a decreased uptake of $^{14}C$ leucine in L cells or HeLa cells (GRANGER et al., 1969).

### 5. Clonal Inhibition

C57BL spleen cells sensitized to DBA/2 mastocytomas inhibit the cloning ability of target cells when mixed in suspension culture (BRUNNER et al., 1966). This clonal inhibition is a useful and rapid system for studying delayed hypersensitivity; an inhibitory effect can be detected as early as 3 hours. The relationship of this assay to delayed hypersensitivity was suggested by the fact that the clonal inhibition was antagonized by antisera directed against the DBA/2 tumour (BRUNNER et al., 1967). LEBOWITZ and LAWRENCE 1969; and in LAWRENCE and LANDY, eds. 1969, pp. 354–356) described a clonal inhibition assay for use with cells sensitized to soluble antigens. Human tuberculin-sensitive blood lymphocytes released a factor in the presence of

PPD which inhibited the cloning ability of HeLa cells. Actual cell death was not observed, rather rounding up and cessation of division. This may be due to the nature of target cell, or may be an indication that the authors are measuring inhibition of proliferation rather than cytotoxicity. GREEN et al. (1970) have described a factor (PIF) released from lymphocytes after interaction with phytohemagglutinin. This factor at a 1 to 20 dilution caused a decrease of DNA synthesis in a variety of cell types including HeLa, human amnion, Hep2 and KB. This work suggests that cell death may be the most extreme form of an activity produced by the supernatant. However, histologically, in the delayed skin reaction, one sees actual cell death and necrosis. Proliferation inhibition and clonal inhibition may be dilution results of the cytotoxic activity described in Section C, lymphotoxin (LT) may be a non-physiologic concentration of PIF, or PIF and LT may be distinct substances with separate functions.

### 6. Isotope Release

Isotope release from target cells has become a popular method of measuring cytotoxicity as indicated in Tables 5, 6, and 7, and has been reviewed by PERLMANN and HOLM (1969). The target is prelabelled with a radioactive precursor which is incorporated into protein, RNA, or DNA depending on the isotope. One of the most frequently employed is Chromium 51. The exact location of this particular label is not clear but PERLMANN and HOLM (1969) state that it is "noncovalently bound to proteins and other cell constituents". Sensitized lymphocytes are mixed with prelabelled target cells. If lysis occurs, the isotope is released into the supernatant where it can be measured. One advantage in interpretation of results of this method is that only lysis of target cells is measured and not inhibition of proliferation or inhibition of metabolism. This should make interpretation in a quantitative sense easier since one has firmer parameters with which to deal. This advantage (i. e. only lysis is recorded) is a theoretical disadvantage in that it renders the method less sensitive than those in which counting of cells or colonies is measured. Thus, only the most extreme form of cell damage (death) is recorded. However, the system appears very sensitive and can be read very soon after the addition of lymphocytes.

The method of isotope release suffers from several other serious disadvantages. Kinetic studies are difficult due to the high background, which rises in the course of time as the result of leakage of label from undamaged cells. Thus BRUNNER et al. (1970) state that at 17 hours, more than 50% of the label ($^{51}Cr$) is released from target cells that are incubated in the absence of lymphocytes. This high background may be dependent, to some extent, on the nature of the target cell. PERLMANN et al. (1968) using fowl erythrocytes were able to reduce the spontaneous release of isotope at 24 hours to less than 10%, and even after 40 hours it was rarely more than 20%. This however is still a fairly high background and makes experiments with slight effects difficult

to assess. Reutilization of the label is believed not to be a problem with $^{51}Cr$, though it is with many other isotopes such as $^{32}P$.

## 7. Discussion

Disagreement and controversy have arisen over the existence and importance of soluble cytotoxic factors in the supernatant of cell cultures in which antigen and sensitized cells have interacted. Thus, cytotoxic factors have been reported in the supernatants when the effects are measured by decrease in cell counts or cell function (RUDDLE and WAKSMAN, 1968; HEISE et al., 1968; GRANGER and WILLIAMS, 1968; DUMONDE et al., 1969; LEIBOWITZ and LAWRENCE, 1969; GREEN et al., 1970), but they have not been described in systems in which $^{51}Cr$ release is measured and actual lysis must occur. Consequently, cytotoxic effects may actually be due to inhibition of proliferation. The time of assay is crucial. RUDDLE and WAKSMAN (1969c) observed their cultures 72 hours after addition of supernatant. Investigators using chromium release as an indication of lysis harvest much earlier than this, due to the leakage from normal cells, which by 20 hours is so high that meaningful experiments cannot be performed. Confusion also has been caused by the presence of polymorphonuclear leukocytes in peripheral blood and spleen, and macrophages in peritoneal exudates. Both cell types cause non-immunologic cytotoxic effects and release toxic factors into culture supernatants. Preparations of "sensitized lymphocytes" with contaminating polymorphonuclear leukocytes and macrophages cells kill fibroblasts in both the presence and absence of antigen. Obviously it is extremely important to use pure cell populations and include antigen specificity controls. The nature of the target cell is another important factor in the cytotoxic systems. The mechanism of susceptibility to lysis has not been established. It is quite clear that L cells for example are more sensitive than any other target cells that have been used.

It has not been resolved whether contact between target cell and effector lymphocyte is necessary for cytotoxicity to occur. One would more likely expect contact to be necessary in the homograft situation, since the antigen is a component of the target, and contact would be necessary for the initial specific reaction of antigen with sensitized lymphocyte. In the systems which involve soluble antigens, if a factor is released, one would not expect contact to be necessary, just as contact is unnecessary in the MIF system once the initial interaction with antigen has occurred. If contact between sensitized lymphocytes and target is unnecessary after interaction with antigen *in vivo*, one would expect to see non-specific destruction in the area of graft rejection. However, the bulk of evidence concerning homografts does not support the concept of a non-specific mediator as responsible for damage in the environment. Contact of sensitized lymphocytes with antigen produces only destruction of specific target cells that contain the antigen. Thus autografts inserted into much larger allografts survive despite rejection of the incompatible graft

(MEDAWAR, 1969), and small numbers of tumour cells mixed with larger numbers of incompatible tumour cells in mice will grow (BENNETT, 1965).

MINTZ and SILVERS (1970) used allophenic mice in an approach to this problem. Skin from such mice, which are derived from aggregating blastomeres of two H-2 incompatible individuals, contains melanoblasts and hair follicle cells of both strains. When such skin was grafted onto one of the "parental" strains, phenotype-specific homograft rejection occurred i.e. in most instances only the cells of nonparental origin were destroyed, and the melanoblasts and hair follicle cells of parental origin survived. Some non-specific destruction was observed, but it appeared to be secondary to necrosis.

Evidence which supports the concept of non-specific destruction in the region of an immune response comes from the work of ELKINS (1964, 1966) and ELKINS and GUTTMAN (1968). ZBAR et al. (1970) showed that a delayed reaction to tuberculoprotein or to a tumour would suppress the growth of another antigenically different tumour, though close contact between sensitized cells, sensitizing antigen, and antigenically unrelated tumour was necessary. This destruction was observed in an already sensitized animal where mediator could be in a greater quantity or could even be qualitatively different. It is conceivable that once the lymphocyte has reacted with surface antigen on target cells, the cytotoxic factors may not diffuse to neighboring cells. Also, the cytotoxic material may be more potent complexed with antigen, and would be most effective against target cells that contain the antigen.

The problem of specificity has not been solved in tissue culture cytotoxicity experiments. The concept in most systems of delayed hypersensitivity has been that sensitized lymphocytes are activated by antigen in a first step which is highly specific. Release of a mediator occurs in a second step. The action of this mediator is not antigen-specific; it inhibits the migration of normal macrophages if it is MIF, causes blastogenesis of normal lymphocytes if it is BF, and kills innocent bystander cells if it is LT. Though BENNETT and BLOOM (1967) originally suggested that MIF was most effective in the presence of specific antigen and AMOS and LACHMANN (1970) demonstrated inhibition of macrophage migration by antigen-antibody complexes, MIF elutes from Sephadex columns with a molecular weight of 35000–55000, which is too small to be complexed with antigen (REMOLD et al., 1970), and is considered a non-specific molecule.

Specificity has been demonstrated in certain *in vitro* experiments which measure homograft cytotoxicity. GINSBURG and SACHS (1965) and GINSBURG (1968) placed normal rat lymphocytes on mouse monolayers of different strains. After allowing sensitization to occur, the lymphocytes were placed on fresh monolayers. The rat lymphocytes destroyed mouse cells and could even distinguish between H-2 specificities. SOLLIDAY and BACH (1970) also observed specificity after *in vitro* sensitization of human cells. Lymphoctes were mixed for 5 days with a particular lymphoblastoid cell line which was treated with mitomycin. When the lymphocytes were added to cultures of sensitizing lymphoblastoid cells, greater target cell destruction as measured

by $^{51}Cr$ release was observed than from other lymphoid cell lines. These experiments demonstrate that specificity occurs, but do not rule out lack of specificity as well, as the "nonspecific" target cells released almost as much label as the "specific" target. This could be explained by cross reactivity of the lines.

Other work has emphasized the non-specific effects of activated lymphocytes. Sensitized cells, that were incubated with antigen for very short periods of time, and then washed, destroyed syngeneic monolayers (RUDDLE and WAKSMAN, 1968c). Supernatants also did the same thing. Though it is possible that antigen remained complexed with the lymphocyte or in the supernatants, column-purified LT induced by PHA has a molecular weight of only 90000–150000 for the mouse and 80000–90000 for human (KOLB and GRANGER, 1970). This is too small to contain an entire molecule of phytohemagglutinin. The experiments of COHEN and FELDMAN (1971) in a homograft system support the concept of the occurrence of nonspecific cytotoxic effects. Rat lymph node cells sensitized *in vitro* are able to damage both specific and non-specific target fibroblasts (as measured by $^{51}Cr$ release). The damage to non-specific fibroblasts by sensitized lymphocytes was augmented significantly in the presence of specific fibroblasts. These experiments are in accord with the postulate that of the occurrence of a primary specific step that results in activation of the sensitized lymphocyte by specific antigen and then a second non-specific step in which the lymphocyte damages innocent bystander cells.

Though most workers agree that those components of complement which are inactivated by heating at 56° are not necessary for cell-mediated cytotoxicity, later steps in the complement cascade have not been ruled out (PERLMANN et al., 1969).

The relationship of cytotoxic systems to the *in vivo* situation is still not entirely clear. Tissue destruction often occurs in delayed reactions, but it has not been established whether sensitized lymphocytes, macrophages or a combination cause the damage. GERSHON and HENCIN (1971) demonstrated the importance of the activation and destruction of macrophages in the delayed skin reaction. Mice do not normally manifest a significant delayed skin reaction. However, if normal peritoneal exudate cells are injected into the skin test site of sensitized mice, a delayed reaction does occur. This reaction is significantly larger in the presence of specific antigen, when actual damage of the macrophages is observed.

Further evidence for the importance of lymphotoxin in the animal comes from work recently referred to in LANCET (1970): Leishmania organisms are sequestered in macrophages in infected animals, subsequently, the antigen leaks out and sensitizes lymphocytes, which then come and destroy the macrophages that still contain the specific antigen.

Table 5. *Cytotoxic and inhibitory methods-Homograft*

| Author (date) | Effector cells | Antigen | Target | Assay |
|---|---|---|---|---|
| WEAVER (1958) | C57 Bl/6 mice, macrophages, lymphocytes | DBA/2 thymoma | DBA/2, ascites cells | direct observation, destruction of tumour cells |
| GOVAERTS (1960) | dog, thoracic duct | Kidney graft | donor kidney cells in culture | direct observation, cytotoxicity |
| MERRILL et al. (1960) | rabbit, peritoneal exudate | skin graft | donor peritoneal exudate cells | viability decrease measured with trypan blue and eosin |
| ROSENAU and MOON (1961) | BALB/C mice, spleen | L cells | L cells | direct observation, lysis and cell damage |
| TAYLOR and CULLING (1963) | BALB/C mice, guinea pigs, spleen | L cells | L cells, guinea pig fibroblasts | Viability counts with trypan blue |
| WILSON (1963, 1965a, 1965b) | Lewis rat, lymph node, thoracic duct | BN skin graft | BN kidney<br>BN tumour cells | Viable counts and Coulter counter |
| VAINIO et al. (1964) | A, SW, DBA, CB57Bl mice, lymph node | skin, tumour, spleen, liver, kidney from A. SW, DBA and C57B1 mice | fibroblasts of donor origin | $C^{14}$ release |
| GRANGER and WEISER (1964) | C57 B/6K mice peritoneal exudate | A/JAX ascites tumour, SAI or A/JAX spleen cells | normal macrophages, fibroblast monolayer of origin of antigen donor | plaques (due to cell bound antibody) |
| BRONDZ (1964) | A, C57 Bl/10Sn, D2, C57L, C3H/Sn, CC57Br mice, lymph node, spleen | SAI sarcoma (of A origin) Mx5 sarcoma (of C57Bl/10 Sn, origin) | sarcoma and macrophage monolayer of donor origin | destruction, counted cells in eosin and trypan blue |
| BRUNNER et al. (1966) | mouse (C57Bl), spleen | DBA/2 mastocytoma | DBA/2 mastocytoma | clonal inhibition |

| | | | | |
|---|---|---|---|---|
| Ulrich and Kieler (1969) | $C_3H$/Fib mice, lymph node | hyperimmunization with tissue culture cells. C3H-M spleen C3H-E embryo culture C3H-L lung | target antigen in Linbro plates | micromethod counted after trypsinisation |
| Brunner et al. (1970) | C57B1/6 mice, spleen | DBA/2 mastocytoma | DBA/2 embryonic fibroblasts, lymphomas lymphocytes | $Cr^{51}$ release |

Table 6. *Cytotoxic and inhibitory methods—Soluble antigens*

| Author | Effector cells | Antigen | Target | Assay |
|---|---|---|---|---|
| Gangarosa et al. (1955) | human, macrophages and fibroblasts from tuberculin-sensitive donors | PPD | same as effector | direct observation, inhibition of proliferation, cytolysis |
| Shea and Morgan (1957) | guinea pig, splenic macrophages from tuberculin-sensitive donors | tuberculoprotein | same as effector | direct observation, migration inhibition and cytotoxicity |
| Glasgow and Morgan (1957) | guinea pig, spleen from infected animal | mumps virus | same as effector | direct observation, migration inhibition and cytotoxicity |
| Tunçman and Packalén (1959) | guinea pig, lung and spleen from tuberculin-sensitive donors | PPD | same as effector | inhibition of growth of tissue from tuberculin-sensitive animals |
| Heilman et al. (1960) | guinea pig, spleen from Brucella-infected donors | Brucella | same as effector | direct observation, inhibition of growth and migration |
| Pincus et al. (1963) | guinea pig, peritoneal exudate | BSA BGG PPD | KB HeLa Hep-2 human amnion, bovine embryonic, epithelial cells, monkey kidney cells | decreased pH |

Table 6. (Continued)

| Author | Effector cells | Antigen | Target | Assay |
|---|---|---|---|---|
| HOLM and PERLMANN (1967) | human, peripheral blood | PPD<br>*staphylococcuss aureus* | Chang liver cells | $Cr^{51}$ release |
| RUDDLE and WAKSMAN (1967, 1968a, b, c) | rat, lymph node | PPD<br>Egg albumin<br>BGG<br>Pic HSA | rat fibroblasts | direct observation, Coulter counter, changes in acid phosphatase, factor produced |
| LUNDGREN et al. (1968) | human, peripheral blood | PHA<br>antigen-antibody complexes,<br>*Salmonella*,<br>mixed lymphocytes | sheep or human fibroblast | Plaques |
| SPEEL et al. (1968) | mouse, spleen | mumps virus | Chang cells (carrying virus) | Coulter counter |
| PERLMANN et al. (1968) | fowl, blood | PHA | erythrocytes | $Cr^{51}$ release |
| DUMONDE et al. (1969) | guinea pig, lymph node | BGG | L cells | counting, cytotoxic factor produced |
| HEISE et al. (1968); HEISE and WEISER, (1969) | guinea pig, lymphocytes | PPD | L cells | direct observation<br>cytotoxic factor produced |
| GRANGER et al. (1969) | human, peripheral blood<br>mouse, spleen, guinea pig, spleen | PPD | L cells<br>HeLa cells | inhibition of uptake of $^{14}C$ labelled amino acids, cytotoxic factor produced |
| LEBOWITZ and LAWRENCE (1969) | human peripheral blood | PPD | HeLa cells | reduction in cloning efficiency,<br>cytotoxic factor produced |

| | | | | |
|---|---|---|---|---|
| Henney (1970) | guinea pig, spleen | DNP-HGG | mouse mastocytoma cells coupled to DNP-HGG | $Cr^{51}$ release |
| Pincus (1970) | guinea pig, lymph node, spleen, peripheral blood | PPD, BSA, tricophyton epidermatophyton | L cells | eosin uptake, cytoxic factor |

Table 7. *Cytotoxic and inhibitory methods—Autoallergies*

| Author | Effector cells | Disease | Target | Assay |
|---|---|---|---|---|
| Perlmann and Broberger (1962) | human, peripheral blood | ulcerative colitis | sheep red blood cell coated with extract of human colon mucosa | $P^{32}$ or $C^{14}$ release |
| Koprowski and Fernandes (1962) | rat, lymph node | experimental allergic encephalomyelitis | puppy brain | direct observation, destruction of gial cells |
| Rose et al. (1963) | rabbit, lymph node | autoimmune thyroiditis | thyroid cells | direct observation, destruction of cells |
| Hedberg and Källén (1964) | human, mononuclear cells from synovial fluid | systemic lupus erythematosus, rheumatoid arthritis, psoriatic athropathy | human embryo, skin fibroblasts | direct observation, destruction of cells |
| Winkler (1965) | rat, lymph node | experimental allergic neuritis | fetal rat, trigeminal ganglion | direct observation, demyelination |
| Seiden (1967) | rat, peritoneal exudate | experimental allergic encephalomyelitis | HeLa or L cells coated with encephalitogen | $C^{14}$ release |
| Ellison et al. (1970) | rat, lymph node | experimental allergic encephalomyelitis | rat fibroblasts (in the presence of basic protein) | Coulter counter, cell destruction |

## VIII. Summary and New Directions in the Assessment of Delayed Hypersensitivity *in vitro*

Sensitized lymphocytes react *in vitro* with antigen and:

1. undergo blast transformation: DNA, RNA, protein synthesis;
2. cause blast transformation in other lymphocytes (BF);
3. inhibit macrophage migration: (MIF, MAF);
4. activate macrophages;
5. release interferon;
6. become cytotoxic (LT);
7. allow virus replication;
8. produce leukotactic factor;
9. prevent macrophage spreading;
10. prevent proliferation (PIF, IDS).

The relationship of the systems described above to each other is still unclear. The difficulty lies in the range of target cells and effector cells, and differences in evaluation of changes in the targets. Many of the activities are produced under the same conditions and could actually be due to the same molecule. HEISE et al. (1968) demonstrated that fluids that contain MIF from the guinea pig are also cytotoxic for fibroblasts. SPITLER and LAWRENCE (1969) showed blast transformation and MIF production in the same cultures, and DUMONDE et al. (1969) described the concurrent production of four different lymphokines. There are also indications that the factors are separate entities: MIF and chemotactic factor are distinct (WARD et al., 1970), interferon is definitely a separate molecular entity, and some physical characteristics of the mediators, such as heat stability, differ.

A tentative scheme is proposed here to relate the mediators of cellular immunity observed *in vitro* to the *in vivo* situation and to each other. This scheme contains assumptions that are actually the basis of much current research, and is presented here only as a working hypothesis. The proposal is an outline of the events at a skin test site in a sensitized animal:

Sensitized lymphocytes which are in the circulation react with antigen at the skin test site and undergo blast transformation. RNA and protein synthesis are necessary for DNA synthesis and cell division. Cell division permits amplification of the population as does blastogenic factor. Lymphotoxin damages cells at the skin test site, which elicits the accumulation of nonspecific mononuclear cells. The mononuclear cells also arise in response to chemotactic factor and remain in part due to migration inhibitory factor. The mononuclear cells become activated or damaged; their lysosomal enzymes cause severe tissue destruction and necrosis. The inhibitor of DNA synthesis and the proliferation-inhibitory factor prevent the reaction from continuing indefinitely by acting as repressors on the lymphocytes, preventing further recruitment and mediator production.

An important area of current investigation is the biochemical analysis of the mediators themselves. This approach should ultimately indicate whether any of the substances produced in the diverse systems are identical. Once the biochemistry of the mediators has been elucidated, it should be possible to detect these substances *in vivo* and determine their relevance to skin graft rejection and delayed skin reactions.

One approach to the quantitation of delayed hypersensitivity is the identification and enumeration of the actual sensitized cells that respond to antigen *in vitro*. As yet it has not been possible to make an antibody against any of the soluble mediators, couple it with fluorescein and visualize those cells containing the mediators, as has been possible with cells making immunoglobulin. Problems in quantitation that are confused in some systems by recruitment, could be approached in this way. An important system recently described by BLOOM et al. (1970) does analyze delayed hypersensitivity at the sinlge cell level presumably before recruitment can occur. This work is based on the observation that lymphocytes activated by PHA or specific antigen can support RNA virus replication. Lymphocytes from tuberculin-sensitive guinea pigs in the presence of PPD allow replication of Newcastle disease virus or Vesicular stomatitis virus. When the lymphocytes are plated in agar in the presence of specific antigen over a monolayer of L cells or chick fibroblasts which are susceptible to the virus, a plaque in the monolayer occurs below those lymphocytes that have supported viral replication. This assay has been demonstrated to correlate with delayed hypersensitivity.

*In vitro* systems of delayed hypersensitivity can be used as tools in the investigation of the immune response. The choice of a method depends on the individual investigator. Obviously those systems whose quantitation is precise and meaningful and which can be related to individual cells will probably prove the most useful. Questions as to whether delayed hypersensitivity is subject to feedback inhibition, what controls its specificity, the nature and origin of cells that participate in the response, are all topics that can be investigated using these systems. Control mechanisms in delayed hypersensitivity and antagonism between different phases of the immune response can be probed with new *in vitro* techniques. HELLSTRÖM and HELLSTRÖM (1970), for example, have demonstrated that antibody from tumour-bearing animals prevent the *in vitro* cytotoxic effect of their own lymphocytes; this work will no doubt be extended to other systems of delayed hypersensitivity as well.

Identification and investigation of effector cells is possible with the use of antigen and chromosome markers. CEROTTINI et al. (1970) injected spleen, thymus or bone marrow cells into irradiated mice of a different strain (DBA/2). After 5 days, cells from the recipient spleens which were presumablythe produce of interaction of injected cells with host antigen, were reacted *in vitro* with DBA/2-derived mastocytoma cells and target cell death was measured by $^{51}Cr$ release. Spleen cells were most effective in reconstituting the response,

as were thymus cells. Bone marrow cells varied depending on the strain. RUDDLE et al. (1971) have investigated the role of thymus-derived and bone marrow-derived populations in a system measuring cytotoxicity in the presence of soluble antigens using histocompatibility antigens as markers. They also observed a surprisingly high reconstitutive ability of bone marrow cells. BRUNNER et al. (1970) have demonstrated the importance of a population of theta antigen-carrying cells (presumably thymus derived) in a cytotoxic system. Investigations at the single cell level should prove even more interesting, particularly since problems of cell-cell interaction, which are currently under intense investigation in the field of immunoglobulin production, are now becoming amenable to analysis in delayed hypersensitivity through the use of quantitative *in vitro* approaches. Finally, the identification of putative antigen receptors on the surface of lymphocytes may be aided by the use of such *in vitro* methods.

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# The Genus Yersinia: Biochemistry and Genetics of Virulence

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With 3 Figures

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# I. Introduction

> "In the autumn of 1665 the spread of the Great Plague caused the closing of the University until the spring of 1667. During those 18 months Newton (sent home from Cambridge) laid the foundations for his famous discoveries in mathematics and physical science."
>
> Encyclopedia Britannica, 1970 Edition

## A. Objectives

A highly successful *modus operandi* in research is to perform an experiment and then decide what problem was solved; the alternative procedure of devising experiments in order to answer preexisting questions may be more difficult. Unfortunately, the latter approach cannot be avoided by those concerned with infectious diseases because the question of why a given microorganism is virulent is automatically framed by its discoverer — in this case, the plague bacillus by YERSIN (1894) and the causative agent of pseudotuberculosis by MALASSEZ, and VIGNAL (1883). The delay of almost a century in obtaining satisfactory answers to these questions simply illustrates that the complexity of the host-parasite relationship is formidable and that experiments relevant to the expression of virulence have not been designed. At the same time, considerable progress towards this end has been made with other pathogens, especially in correlating nutritional requirements and metabolic patterns with the bacterium's favored habitat *in vivo* (MOULDER, 1962). Nevertheless, the diverse phenomena observed during infection with intracellular parasites cannot always be duplicated in simplified experimental systems. Furthermore, the infectivity of many microorganisms is low or erratic in laboratory animals and the response of the normal host to acute and chronic phases of disease may be quite distinct.

These difficulties are minimized to some extent by the experimental approach used in studies with *Yersinia pestis*, the causative agent of bubonic plague. This organism, which is highly infectious and lethal in most rodents and primates, yields well-characterized avirulent mutants at known rates. The products affected by the altered genes in these mutants are, by definition, directly associated with the pathogenic process; those which function during intracellular growth are evidently distinct from those associated with invasion and extracellular survival. Furthermore, plague is usually terminated by death of the host or elimination of the parasites following acquisition of humoral immunity. Accordingly, the interesting, but ancillary, problem of chronic disease seldom exists although the infection caused by closely related *Y. pseudotuberculosis* is often chronic in nature.

Unique information to be gleaned from the study of *Y. pestis* therefore relates principally to an uncompromising process of bacterial invasion and to the role of nonspecific mechanisms of host-defense. The problem of invasion

will be treated in some detail in this review where the intent is to emphasize biochemical and genetic determinants of virulence rather than the development of immunity. Much of our current understanding of both of these topics is largely based upon the findings of T. W. BURROWS and co-workers; the reader is invited to assess the extent of their contribution by comparing the information contained in the reviews of BURROWS (1960, 1962, 1963) with that available a few years earlier (GIRARD, 1955). The epidemiology of plague, cultural and biochemical properties of *Y. pestis*, and related phenomena have been discussed in detail (POLLITZER, 1954; TUMANSKII, 1958; BALTAZARD, 1963) and summaries of work in selected areas relating to virulence have appeared (SURGALLA, 1960; KADIS et al., 1966; WALKER, 1967; SURGALLA et al., 1968). Comprehensive reviews of the Russian literature were prepared by POLLITZER (1966) and DOMARADSKII (1966).

## B. Taxonomy

In accord with the proposals of SMITH and THAL (1965) and KNAPP (1965), the subcommittee on *Pasteurella*, *Yersinia*, and *Francisella* provisionally recommended at the Xth International Congress of Microbiology (1970) that the proposed genera *Yersinia* (VAN LOGHEM, 1945) and *Francisella* (DOROFEEV, 1947) be used for classification of those species placed, according to *Bergey's Manual* (7th Edition), in the genus *Pasteurella*, but that the latter genus be retained for disposition of the type species *P. multocida* (*septica*). The genus *Yersinia* contains *Y. pestis* and *Y. pseudotuberculosis* which share at least a dozen antigens (BHAGAVAN et al., 1956; RANSOM, 1956; THAL, 1956; LAWTON et al., 1960; BURROWS and BACON, 1960; LARABEE et al., 1965), exhibit similar sensitivies to certain bacteriophages (STOCKER, 1955; SMITH and BURROWS, 1962), and possess nearly homologous DNA (RITTER and GERLOFF, 1966). Tentatively included is a recently discovered organism termed *Y. enterocolitica* which has not yet been fully characterized; this organism is discussed briefly in section VI. *Y. pestis* and *Y. pseudotuberculosis* are only distantly related to *P. multocida* and the Francisellae (*F. tularense* and *F. novicida*) as judged by heterology of DNA (RITTER and GERLOFF, 1966) and other criteria (PHILIP and OWEN, 1961) which indicate retention of the latter in the *Brucellaceae*.

A taxonomic relationship between the yersiniae and enteric bacteria was first suspected on the basis of common sensitivities to phages (GIRARD, 1943; LAZARUS and GUNNISON, 1947; STOCKER, 1955; SMITH and BURROWS, 1962) and similarities detected by Addisonian analysis (SNEATH and COWAN, 1958). A closer examination of the common phages disclosed the presence of antigens shared by T2 and T3 coliphages (HERTMAN, 1964; ACKERMANN and POTY, 1969) and a host-range mutant of T6 coliphage was obtained which lysed cells of *Y. pseudotuberculosis* (*unpublished observations*). Other studies showed that yersinia are capable of accepting F-*lac*$^+$ (MARTIN and JACOB, 1962) and RTF (GINOZA and MATNEY, 1963; KNAPP and LEBEK, 1967) from *Escherichia coli*. BRUBAKER and SURGALLA (1961) and SMITH and BURROWS (1962) also noted

that pesticin I, a bacteriocin-like substance produced by *Y. pestis*, kills cells of certain strains of *E. coli* including the universal colicin indicator strain $\phi$. Subsequently, RITTER and GERLOFF (1966) demonstrated a 22% hybridization between fragmented DNA of *E. coli* and whole DNA of *Y. pestis*. LAWTON et al. (1968) noted that *E. coli* shares at least 3 antigens with *Y. pseudotuberculosis* as judged by gel-diffusion analyses. The guanine plus cytosine content of DNA obtained from *Y. pseudotuberculosis* is 45.6% (LAWTON et al., 1968); this value is similar to that reported for *Y. pestis* but about 4% lower than that determined for *E. coli* (MARMUR et al., 1963). These findings are generally in accord with the recommendation, which is supported by this reviewer, to place the genus *Yersinia* within the Family *Enterobacteriaceae*.

# II. Yersinia pestis

## A. Physiology

Properties discussed in this section are generally common to both virulent and avirulent cells. Factors associated with pathogenicity are considered in subsequent sections (II. B., II. C.).

Strains of *Y. pestis* isolated from widely spaced geographical locations exhibit remarkably similar characteristics. There are no recognized phage-types or serotypes as exist for other members of the genus although DEVIGNAT (1951) distinguished 3 varieties or biotypes on the basis of their ability to accumulate $NO_2^-$ from $NO_3^-$ and to ferment glycerol. Variety *orientalis* forms $NO_2^-$ but fails to ferment glycerol, variety *antiqua* forms $NO_2^-$ and ferments glycerol, and variety *mediaevalis* fails to form $NO_2^-$ but ferments glycerol. Variety *orientalis* is currently endemic in India, southwest Asia, South America, and western North America; variety *antiqua* is also present in southeast Asia as well as Manchuria, northern China, and areas of Russia and Africa. Variety *mediaevalis* is limited to endemic areas of Turkey, Iran, and foci adjacent to the Caspian Sea. TUMANSKII and URIUPINA (1960) recognized varieties of *Y. pestis* isolated in the Soviet Union on the basis of geographic distribution and host preference.

### 1. Nutrition

Growth of *Y. pestis* occurs at temperatures between −2° C and 45° C but the fastest rate is observed in usual cultural media at about 28° C (SOKHEY and HABBU, 1943a). Although the optimal pH is between 7.2 and 7.6, the organisms tolerate extremes of 5.0 to 9.6 (SOKHEY and HABBU, 1943b). Even at room temperature, long generation times of 2 hours or more are observed during logarithmic growth in enriched media. The final yield of cells in such media, however, may approximate $10^{11}$ per ml (HIGUCHI and CARLIN, 1957). Cells of *Y. pestis* and *Y. pseudotuberculosis* are especially sensitive to high concentrations of free amino acids, such as those in the media of ROCKENMACHER et al.

(1952) or HIGUCHI and CARLIN (1958), during aeration at 37° C (C. E. LANKFORD, *personal communication;* BRUBAKER, 1967). This effect can be minimized, depending upon the purpose of the medium, by substitution of peptides for amino acids, use of heavy inocula, dilution of the amino acid component, or incubation without aeration. For example, inocula greater than $10^8$ cells per ml are necessary to initiate growth at 37° C in the rich synthetic media of HIGUCHI and CARLIN (1958) and HIGUCHI et al. (1959) but these media eventually yield over $10^{10}$ cells per ml. In contrast, an inoculum of $10^4$ to $10^5$ cells per ml is sufficient to initiate growth in the well-balanced medium of BROWNLOW and WESSMAN (1960) but the final crop of cells seldom exceeds $10^8$ per ml. Partial enzymatic digests and acid-hydrolysates of protein or infusions of natural products are routinely used for cultivation at 37° C when chemically defined media are not required. The utilization of glycyl-peptides by cells of *Y. pestis* was investigated by SMITH and HIGUCHI (1959). The individual amino acids which account for inhibition of growth at 37° C have not yet been identified but C. E. LANKFORD noted that toxicity is a function of high oxygen tension and can be eliminated by the addition of D-alanine; these observations were verified by BRUBAKER (1967). A toxic effect of D-serine can be reversed by glycine, purines, L-serine, and glyoxylate (SMITH and HIGUCHI, 1960).

Hemin and mercaptoacetate, shown by HERBERT (1949) to favor the formation of colonies on solid media, can be replaced by a number of other reducing agents (HILLS and SPURR, 1952; BURROWS and GILLETT, 1966) and, in liquid medium, by potential precursors of hemin (BROWNLOW and WESSMAN, 1960). Oxidizable organic acids have been employed in synthetic media with favorable results (JACKSON and BURROWS, 1956a; HIGUCHI and CARLIN, 1958; BRUBAKER, 1970) and high concentrations of $Fe^{+2}$ (0.5 mM) stimulate cell division in the medium of HIGUCHI and CARLIN (1957). At 26° C, cells of *Y. pestis* typically exhibit a nutritional requirement for L-methionine and L-phenylalanine; growth is enhanced by addition of L-isoleucine, L-valine (HILLS and SPURR, 1952; ENGELSBERG, 1952) and glycine (BURROWS and BACON, 1954). According to BURROWS and GILLETT (1966), strains of the variety *mediaevalis* lack the requirement for L-phenylalanine but it should be noted that all phenylalanine-independent isolates examined by these workers were of this variety. Glycine may be replaced by L-threonine (JACKSON and BURROWS, 1956a; BRUBAKER and SULEN, 1971). Exogenous biotin is necessary for growth at 36° C and, at 38° C, the organisms also require panthothenate, thiamin, and glutamic acid; the latter can be replaced by α-ketoglutarate (BROWNLOW and WESSMAN, 1960). Using a solid medium of similar composition, BURROWS and GILLETT (1966) could promote the formation of colonies at 37° C in the absence of added biotin and panthothenate provided that the organisms were incubated in a $CO_2$-enriched atmosphere.

Low concentrations of $NH_4^+$, supplied at constant rates by diffusion through agar, spontaneous hydrolysis of urea, or oxidation of amino acids, are unable to serve most isolates of *Y. pestis* (termed $N^-$) as primary sources of nitrogen. However, cells of a few strains (designated $N^+$) are able to assimilate low levels of

$NH_4^+$; both $N^+$ and $N^-$ organisms can grow equally well in the presence of high (0.01 M) concentrations of $NH_4^+$. No correlation was noted between biotype of DEVIGNAT and expression of the $N^+$ phenotype (BRUBAKER and SULEN, 1971). Cells of *Y. pestis* form L-cysteine from exogenous $S^{-2}$, $S_2O_3^{-2}$, or $SO_3^{-2}$ but not $SO_4^{-2}$ or L-methionine (ENGLESBERG, 1952). Growth *in vitro* is dependent upon the presence of a fermentable carbohydrate which can be any of a number of common hexoses, pentoses, or polyols (POLITZER, 1954). PITAL (1960) showed that the generation time of *Y. pestis* can be dramatically decreased at 37° C by a combination of 4% coconut water and 0.1% albumin fraction V (Dubos oleic-albumin complex). On the other hand, a lipoprotein of host origin (EISLER and von METZ, 1963, EISLER et al., 1967) and certain free fatty acids (EISLER and VON METZ, 1968) are toxic to *Y. pestis*.

## 2. Intermediary Metabolism

*Y. pestis* possesses an operational Embden-Meyerhof pathway (SANTER and AJL, 1955a) but the subsequent claim by SANTER and AJL (1955b) that the cells contain glucose 6-phosphate dehydrogenase could not be verified by MORTLOCK (1962), MORTLOCK and BRUBAKER (1962), BOWMAN et al. (1967), or EISLER and HECKLY (1968); the latter workers also failed to detect β-D-glucose dehydrogenase in *Y. pestis*. Accordingly, glucose cannot be metabolyzed via the hexose-monophosphate pathway and pentose is evidently synthesized via rearrangement of 3 C and 6 C fragments by transketolase and transaldolase DODIN and BRYGOO (1959) noted that rhamnose-adapted *Y. pestis* exhibit a reduced ability to utilize glucose. Gluconate is catabolyzed via an adaptive Entner-Doudoroff pathway and remaining enzymes of the hexose-monophosphate pathway (MORTLOCK, 1962). Xylose isomerase was first purified from extracts of *Y. pestis* (SLEIN, 1962) and other enzymes of pentose conversion are assumed to be analogous to those of enteric bacteria. Cells of the varieties *antiqua* and *mediaevalis* possess nonspecific triose kinase activity (*unpublished observations*) but the actual mechanism for the formation of dihydroxyacetone has not been resolved. DOMARADSKII et al. (1968) suggested that the first step in the catabolism of glycerol is its oxidation to glyceraldehyde. Under anaerobic conditions the primary products of glucose fermentation are lactate, ethanol, acetate, and formate; acetoin is not produced (ENGLESBERG et al., 1954; SANTER and AJL, 1955a).

The existence of a functional tricarboxylic acid cycle in aerated cells of *Y. pestis* was demonstrated by SANTER and AJL (1954) and ENGLESBERG and LEVY (1955). The oxidation of glucose, ribose, lactate, and pyruvate is constitutive in organisms grown *in vitro* at 26° C and 37° C; an adaptive response is obtained with xylose and gluconate but the latter, curiously, is oxidized by cells cultivated *in vitro* (FUKUI et al., 1962). Neither this study nor that of YANG and BRUBAKER (1971a) verified the claim of DODIN and BRYGOO (1960) that the capacity of *Y. pestis*, cultivated at 26° C, to oxidize hexose becomes reduced upon subsequent incubation at 37° C. The organisms contain an ADP-dependent

phosphoenolpyruvate carboxykinase and an irreversible phosphoenolpyruvate carboxylase which catalyze the fixation of $CO_2$ into oxalacetate (BAUGH et al., 1946a).

Aspartase, which was characterized by KOROBEINIK and DOMARADSKII (1968), and a particulate $NADPH_2$-transhydrogenase system (BRUBAKER, 1968), are repressed under conditions that necessitate the primary fixation of $NH_4^+$ into α-amino groups via the action of glutamic acid dehydrogenase. Presumably all 3 of these enzymes are involved in the catabolism of glutamic acid (BRUBAKER and SULEN, 1971). RAO (1940) first showed that serine and alanine are rapidly oxidized by *Y. pestis* and that the oxidation of glutamic acid occurs at a more limited rate. This finding was confirmed by INAMDAR and GANAPATHI (1964) by observing the incorporation of radioactive amino acids into growing cells and by OLENICHEVA and ATAROVA (1968) who also obtained evidence suggesting that *Y. pestis* contains L-amino acid oxidase, asparaginase, and enzyme systems that effect the rapid destruction of ornithine, alanine, and serine; the latter is converted to pyruvate by serine dehydratase as shown by LEVINE et al. (1954). INAMDAR, and GANAPATHI (1964) noted that essentially all of the radioactivity of incorporated $^{14}C$-isoleucine can be recovered as protein as compared to that of other amino acids which are also catabolyzed (L-alanine, D-alanine, DL-serine, and DL-glutamic acid) or converted into lipid (DL-serine, DL-glutamic acid, glycine) and nucleic acid (L-alanine, DL-serine, DL-glutamic acid, glycine) fractions. The data obtained in this work relating to the metabolic fate of glycine is in accord with that previously reported by DOMARADSKII and SEMENUSKINA (1957) who, incidentally, were unable to detect the incorporation of radioactivity from glycine into serine or other amino acids. WESSMAN and MILLER (1966) showed that the arginine pool in *Y. pestis* is rapidly depleted in resting cells.

A block in the conversion of cysteine to cystathionine presumably accounts for the nutritional requirement for methionine (ENGLESBERG, 1952); that for phenylalanine has not been characterized in detail. The biosynthesis of other amino acids, with the possible exception of glycine, evidently occurs by established mechanisms. The production of arginine and pyrimidines may be limited by the formation of carbamyl phosphate, a common intermediate (BAUGH et al., 1964b); reactions catalyzing the subsequent formation and interconversion of uridylic and cytidylic acids have received less attention than have those associated with the *de novo* biosynthesis of purines. According to BEKKER (1967), enzymes of the purine pathway are subject to both feedback inhibition and repression by adenine and guanine. MAISKII (1967) showed that cells of *Y. pestis* are able to convert guanine residues to adenine but lack the capacity to incorporate radioactivity from adenine into guanine. Slow conversion of the latter was detected in a subsequent study (MAISKII, 1968) as was the formation of both guanine and adenine nucleotides from exogenous hypoxanthine and the conversion of exogenous xanthine to guanine. The differences between these reports involving the ability to convert the carbon skeleton of adenine to guanine were shown independently by MAISKII and SUCHKOV (1970) and BRUBAKER (1970)

to relate to a deficiency of adenine deaminase in *Y. pestis*. Due to this metabolic block, the organisms must rely on the histidine cycle to convert exogenous adenine to IMP via aminoimidazolecarboxamide ribotide and, in cases where conversion is not observed, the latter pathway is repressed by exogenous histidine. The ability of *Y. pestis* to complete the *de novo* biosynthesis of purine ribotides is an established determinant of virulence and is discussed further in section II. B. 5.

### 3. Structure

Cells of *Y. pestis* cultivated at room temperature generally appear as short ($1.5 \times 0.5$ μ) ovoid bipolar staining rods which lack flagella. However, depending upon the genotype and mode of cultivation, the organisms may assume a variety of shapes and sizes. The ultrastructure of the normal cell closely resembles that of other typical Gram-negative rods although, depending upon the procedure of cultivation, the cytoplasm may contain electron-dense granules and mesosome-like structures (KATS, 1966; AVAKYAN et al., 1967; YANG et al., 1971b). Standard embedding and fixation procedures are sufficient to effect sterilization of small pieces of tissue (SMIRNOVA et al., 1966) and the envelope or capsular antigen is partially destroyed during preparation for electron microscopy (CROCKER et al., 1956; KATS, 1966). An electron micrograph of a typical dividing cell is shown in Fig. 1.

A controversy as to whether the cells produce a morphologically distinct capsule (SOKHEY, 1940; P'AN et al., 1950; AMIES, 1951) or undifferentiated envelope (ROWLAND, 1914; SCHUTZE, 1939; ENGLESBERG and LEVY, 1954; CROCKER et al., 1956) was critically reviewed by BURROWS (1963) who favored the latter position. Capsular antigen precipitates between 25 and 33 percent saturation with $(NH_4)_2SO_4$ and, according to BAKER et al. (1952), consists of protein complexed with carbohydrate (fraction 1A) and protein alone (fraction 1B). A preparation evidently identical to the polysaccharide portion of fraction 1A was shown by BAKHRAKH et al. (1958) to contain N-acetyl glucosamine and hexuronic acid. These workers also isolated a second polysaccharide of qualitatively similar composition, which, unlike fraction 1A, is shared by *Y. pseudotuberculosis*. Other polysaccharides of uncertain anatomical origin have been examined with respect to allergenic activity (BAKHRAKH and TARANENKO, 1969) and influence on serum proteins (EFIMTSEVA et al., 1968). According to KOROBKOVA and BAKHRAKH, quoted by DOMARADSKII and SEMENUSHKINA (1957), polysaccharide formation is favored by the presence of exogenous glycine. A polysaccharide-containing complex from *Y. pestis* was shown by EFIMTSEVA and VALKOVA (1968) to possess pronounced antitumor activity. Lipopolysaccharide (O-antigen) was isolated by DAVIES (1956) and shown to contain glucose, glucosamine, and an aldoheptose subsequently identified as L-glycero-D-mannoheptose (FOSTER et. al., 1958). According to DAVIES et al. (1958), lipopolysaccharide from *Y. pestis* contains phospholipid which resembles the lipid A of WESTPHAL and LÜDERITZ (1954) obtained from enteric bacteria. Preparations of this type were shown by WALKER et al. (1966)

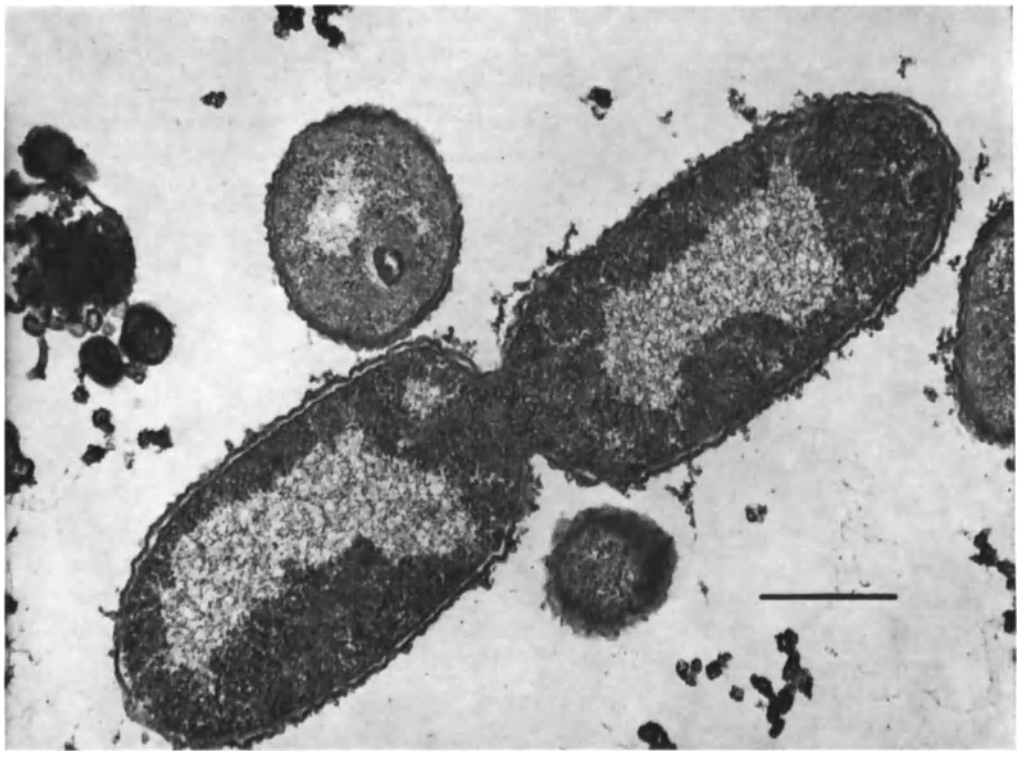

Fig. 1. Electron micrograph of dividing *vwa*$^-$ cell of *Yersinia pestis* strain EV 76 cultivated at 37° C with 0.0025 M $Ca^{+2}$

to contain additional lipid determinants. The chemical configuration of the repeating subunits of lipopolysaccharides from *Y. pestis* have not yet been determined; nevertheless, the organisms are considered to be immunologically rough. Relevant serological studies were performed by DODIN (1963) and DODIN et al. (1964a, 1964b).

Using gas-liquid chromatography in polar and nonpolar solvents, infrared spectroscopy, and ultraviolet spectroscopy, ALIMOVA and BOIKOVA (1967) demonstrated 31 fatty acids (8 saturated, 21 unsaturated, and 2 branched-chain) in the bound lipid fraction. Palmitic, palmitoleic, heptadecaenic, and oleic acids are prominent among the free fatty acids and substantial amounts of branch-chain acids (6,9-hexadecadienic and 11-nonadecaenic) were detected in the bound lipid fraction.

## B. Recognized Determinants of Virulence

By estimating $LD_{50}$ of mutants lacking presumptive determinants of virulence, it is possible to determine directly the contribution of the missing property of the process of infection. Use of this approach has resulted in recognition of the following 5 determinants of virulence: production of viru-

lence or V and W antigens (*vwa*$^+$), fraction 1 antigen (*fra*$^+$), pesticin I (*pst*$^+$), endogenous purines (*pur*$^+$), and expression of a surface structure which absorbs certain aromatic dyes and natural pigments (*pgm*$^+$). The symbols used to define these determinants have been modified from those employed previously in order to conform with the recommendation of DEMEREC et al. (1966). All of these factors can be estimated quantitatively *in vitro* (BURROWS, 1963; SURGALLA, BEESLEY, and ALBIZO, 1970) and undergo independent mutational loss resulting in the degrees of avirulence in mice and guinea pigs shown in Table 1.

Mutations to *pur*$^-$ are generally obtained by treatment with agents known to induce point mutations. As expected, such mutants can often undergo reversion to prototrophy whereas such reversion has not been observed with certainty with the other determinants. Mutation to *vwa*$^-$, *fra*$^-$ and *pgm*$^-$ occurs at high frequency and one or more of these genes may, as first suggested by OGG et al. (1958), reside on an extrachromosomal replicon. However, the only determinant that has been established with some certainty to be under extrachromosomal control is the ability to produce pesticin I; as noted by BURROWS (1965), this factor is retained with tenacity. Studies on the inheritance of virulence have been hindered by lack of a method of efficient gene transfer. LAWTON et al. (1968) were able to exchange certain markers in *Y. pseudotuberculosis* at frequencies of $10^{-4}$ to $10^{-5}$ via F′-*lac* but not all possible classes of recombinants were recovered. Higher frequencies were obtained in subsequent work but the chromosome appeared to be transferred in more than one linkage group (LAWTON and STULL, 1971).

### 1. Virulence or V and W Antigens

BURROWS (1956) used gel diffusion plates to test virulent cells, previously aerated at 37° C, against homologous antisera which had been absorbed with similarly prepared avirulent organisms. A single band was detected which yielded a reaction of identity with other virulence strains and the precipitated antigen was termed "Vi" in anticipation of a role in determining virulence. With the subsequent discovery of a second antigen, termed W, which was produced under identical conditions, the original Vi antigen was redesignated V by BURROWS and BACON (1956). These workers noted that aeration at 37° C in highly enriched medium favored the production of V and W; neither antigen was detected following similar incubation at room temperature. V and W were separated from one another and purified 100-fold and 1000-fold, respectively, by LAWTON et al. (1963) who concluded that V is a protein with a molecular weight of 90,000 and W is a lipoprotein with a molecular weight of 145,000 (38% lipid and 59% protein). Both antigens are always produced together and one is not the precursor of the other.

Following the discovery of V and W, the finding of DEVIGNAT and SCHOETTER (1942) that virulent cultures of *Y. pestis* become attenuated upon serial transfer at 37° C in aerated broth was verified by FUKUI et al. (1957a) who

attributed this change to selection of avirulent mutants. In the latter study it was shown that virulence can be maintained during aeration at 26 C or on solid medium at 37° C. The population-shift to avirulence was prevented by OGG et al. (1958) by adjustment of the initial pH to 7.8, addition of a heat-sensitive substance in spent media, or reduction of oxygen tension. Realizing that a significant concentration of $CO_2$ would be in solution at pH 7.8, DELWICHE et al. (1959) showed that addition of $CO_2$ gas or $NaHCO_3$ to buffered medium can also prevent the rapid outgrowth of avirulent mutants as can agitation of cultures at 37° C under $N_2$ gas. Using a chemically defined medium, WESSMAN et al. (1958) had previously shown that virulent, but not avirulent, cells undergo lysis during aeration at 37° C following the completion of a few divisions in the presence of glucose; this effect can be reduced or eliminated by incubation at 36° C or lower, substitution of xylose for glucose, or addition of $Mg^{+2}$ (0.02 M) or $Mn^{+2}$ (0.002 M). Extending the study of mineral metabolism, KUPFERBERG and HIGUCHI (1958) demonstrated that the growth of virulent cells in aerated cultures at 37° C is strongly dependent upon the presence of $Ca^{+2}$; this observation was refined by HIGUCHI et al. (1959) who showed that virulent cells remain static in the presence of high concentrations of $Mg^{+2}$ (0.02 M) unless $Ca^{+2}$, $Sr^{+2}$, or $Zn^{+2}$ (0.0025 M) is present. Avirulent organisms do not exhibit this magnesium-dependent requirement for $Ca^{+2}$. Taking advantage of the "Ca-requirement" of virulent cells, HIGUCHI and SMITH (1961) developed a solid medium containing 0.02 M $MgCl_2$ and 0.02 M sodium oxalate which is selective at 37° C for avirulent cells. By use of a fluctuation test, the latter were found to arise at the high mutation rate of $10^{-4}$ thus providing an explanation for the rapid shift in population predicted earlier. Mutation from *vwa*$^-$ to *vwa*$^+$ has not been convincingly demonstrated (BURROWS and BACON, 1954).

It was generally known at this time that the avirulent organisms studied by FUKUI et al. (1957b), OGG et al. (1958), WESSMAN et al. (1958), DELWICHE et al. (1959), and HIGUCHI et al. (1959) were of the *vwa*$^-$ genotype (BURROWS, 1960; SURGALLA, 1960) although the first citation of experimental evidence correlating loss of this determinant and Ca-independence is evidently that of BRUBAKER and SURGALLA (1962a). These workers also discovered a rare phenotype, termed "VW$^+$-avirulent" where the requirement for $Ca^{+2}$ but not the ability to produce V and W is lost. These mutants often exhibit a rough colonial morphology on the selective medium of HIGUCHI and SMITH (1961) and are easily isolated at 26° C by selection for resistance to high levels of streptomycin. Resistance to streptomycin is not, however, common to all members of this phenotype which are uniformly avirulent thus suggesting that the expression of Ca-dependence *per se* is correlated with pathogenicity. It is of interest to note that the Russian vaccine strain 1 of *Y. pestis* is of the VW$^+$-avirulent type (*unpublished observations*).

With the introduction of the selective medium of HIGUCHI and SMITH (1961) it became possible to study the population dynamics of cultures containing $Ca^{+2}$ and $NaHCO_3$. SURGALLA et al. (1964) showed that high concentrations of

$NaHCO_3$ (0.125 to 0.15 %) are inhibitory to *vwa*$^+$ and *vwa*$^-$ cells, low concentrations (0.05 to 0.075 %) favor growth of both types, and an intermediate level (0.1 %) selectively inhibits organisms of the *vwa*$^-$ genotype. In contrast, $Ca^{+2}$ (0.002 M) enhanced the rate of growth of *vwa*$^+$ cells. BAUGH et al. (1964b) subsequently showed that known products of $CO_2$ fixation (via carbamyl phosphate) such as orotic acid (0.01 M) or cytosine (0.01 M) also maintain virulent populations during aeration at 37° C. However, the effect of these compounds mimicked that of $Ca^{+2}$ (stimulation of *vwa*$^+$ cells) rather than that of $CO_2$ (inhibition of *vwa*$^-$ cells). SURGALLA et al. (1968) demonstrated that the response obtained in the presence of $Ca^{+2}$ is also elicited to a lesser degree by KSCN, potassium oleate, and sodium deoxycholate whereas that produced by $NaHCO_3$ is mimicked by 2,4-dinitrophenol, KI, sodium salicylate, and biliverdin. These workers noted that all of the active compounds that were tested share the ability to interact with cell surfaces and to cause either uncoupling of oxidative phosphorylation or promotion of ATPase activity. They further suggested that those substances which restrict the proliferation of *vwa*$^-$ cells impose a metabolic disadvantage already present in the *vwa*$^+$ cells and that this disadvantage, assumed to be a disruption of normal membrane function, is in turn relieved by those compounds which act by favoring the growth of *vwa*$^+$ cells.

The addition of $Ca^{+2}$ (0.0025 M) to *vwa*$^+$ cells of *Y. pestis* 2 hours after the onset of stasis results in the initiation of cell division after a further lag of about 2 hours (Fig. 2). However, correspondingly longer lag periods are observed following the addition of $Ca^{+2}$ after 4 and 6 hours of stasis and the final rates of growth become progressively slower. In contrast, reduction of temperature from 37° C to 26° C, without addition of $Ca^{+2}$, results in commencement of rapid growth after a lag of 90 to 120 minutes regardless of the duration of stasis (YANG and BRUBAKER, 1971b).

Studies on the biosynthesis of V and W revealed that production is enhanced in pancreatic digest of casein containing 0.01 M magnesium gluconate; no synthesis occurs in an atmosphere of 100 % $CO_2$, 95 % $N_2$ and 5 % $CO_2$, or *in vacuo*. Lower concentrations of these antigens are detected in the presence of sodium or potassium gluconate and no production is noted upon aeration with calcium gluconate (LAWTON et al., 1963). BRUBAKER and SURGALLA (1964) verified that $Mg^{+2}$ (0.02 M) is essential for optimal production of V and W in aerated broth despite the fact that this addition promotes stasis in Ca-deficient media. In fact, the addition of sufficient $Ca^{+2}$ (0.0025 M) to insure rapid growth of *vwa*$^+$ cells at 37 °C results in complete repression of V and W even in the presence of 0.02 M $Mg^{+2}$. Of 16 tested sources of energy, none was found to enhance the production of virulence antigens in the absence of $Ca^{+2}$ without equally favoring cell division in its presence. Accordingly, a correlation exists between stasis and optimum production of V and W *in vitro*. This correlation accounts for the inability of PIRT et al. (1961) to produce significant concentrations of V antigen by continuous cultivation and underlines the necessity of using batch cultures to obtain this antigen. The growth curves obtained by GADGIL (1964), GADGIL et al. (1967a), and GADGIL et al. (1967b) while studying

the feasibility of cultivating *vwa*$^+$ cells at 37° C for production of vaccine, were generally explained on the basis of population shifts which occur in the absence of sufficient $Ca^{+2}$ to prevent stasis. BURROWS and GILLETT (1966) were unable to detect a significant requirement for $Ca^{+2}$ at 37° C on the selective medium of HIGUCHI and SMITH (1961) or on defined solid media containing 6 amino

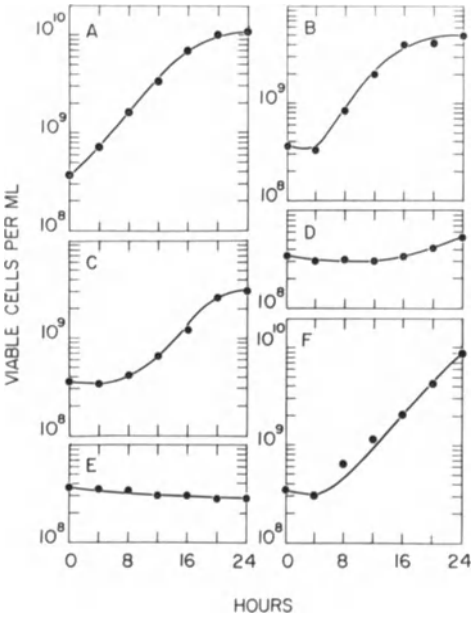


Fig. 2. Response of *vwa*$^+$ cells of *Yersinia pestis* strain EV 76 at 37° C in $Ca^{+2}$-deficient medium to addition of $Ca^{+2}$ (0.0025 M) after aeration for 0 hr (A), 2 hr (B), 4 hr (C), 6 hr (D), and no addition (E); reduction of temperature to 26° C after 6 hr of incubation is shown in F. (After YANG and BRUBAKER, 1971 b)

acids. Evident use of an inoculum containing a high (3 %) population of *vwa*$^-$ cells accounts for part of this difficulty; growth on the defined media may also reflect their inability to promote synthesis of V and W which is prerequisite for the expression of stasis. WEINBERG (1966) considered V and W to be secondary metabolites; TERENT'EVA (1967) found that virulent cells do not require $Ca^{+2}$ during cultivation on solid medium at temperatures lower than 32° C.

BRUBAKER and SURGALLA (1964) observed that static organisms actively synthesizing V and W are elongated and fail to exhibit morphological evidence of invagination or septation. GADGIL et al. (1966) verified this finding and, with some foresight, noted that elongation could occur if the synthesis of DNA was selectively inhibited. The possibility that stasis reflects primary damage to the cytoplasmic membrane, suggested by SURGALLA et al. (1968) and by results obtained with *vwa*$^+$ cells of *Y. pseudotuberculosis* (BRUBAKER, 1967), was not substantiated by BRUBAKER and YANG (1971) and YANG and BRU-

BAKER (1971a) who failed to detect significant differences between dividing and static cells with respect to permeation of L-isoleucine, oxygen uptake, or release of preloaded $^{32}P$. The cytoplasmic membranes of static cells could not be distinguished from those of dividing organisms by inspection of thin sections with the electron microscope (YANG et al., 1971b).

FUKUI et al. (1960) demonstrated a phenotypic loss of virulence during growth at 5° C which can be completely restored by short incubation at 37° C without significant cell division. This phenotypic gain of virulence is correlated with acquisition of certain temperature-dependent properties including the expression of V and W and, as shown by NAYLOR et al. (1961), is dependent upon the presence of oxygen, temperatures between 37° C and 41° C, pH values between 5.5 and 8.4, a mixture of 13 amino acids, $HPO_4^{-2}$, and a source of energy. In a subsequent attempt to define the relation of nucleic acid and protein synthesis to production of V and W, FUKUI et al. (1961) noted that inhibitors that block the synthesis of RNA and protein prevent the phenotypic restoration of virulence. Inhibitors that block the synthesis of DNA, which does not replicate during the short period required for restoration of virulence, are unable to prevent this process.

Significant replication of DNA would not, of course, be expected during maximum synthesis of V and W because the organisms remain static. A reduction in the rate of DNA synthesis, such as that which occurs following a "step-down" or in cells lacking some essential nutrient, is generally accompanied by a decrease in net synthesis of other macromolecules which contribute to cell mass (MAALØE and KJELDGAARD, 1966). However, the apparent increase in cell mass observed by BRUBAKER and SURGALLA (1964) and GADGIL et al. (1966) suggested that significant rates of RNA and protein synthesis might be maintained during stasis. This assumption seemed reasonable in view of the finding that synthesis of RNA and protein are required for the phenotypic restoration of virulence (FUKUI et al., 1961) because their continued production should be necessary for maintenance of this phenotype. BRUBAKER and YANG (1971) and YANG and BRUBAKER (1971a) showed that static cells which had been starved for $Ca^{+2}$ for 12 hours were able to synthesize RNA and protein at rates that were essentially identical to those of dividing *vwa*$^+$ and *vwa*$^-$ cells; as expected, no significant synthesis of DNA occurred in static organisms (Fig. 3).

Further study by YANG and BRUBAKER (1971b) disclosed that the rate of DNA synthesis gradually decreases during stasis with cessation occurring about 4 hours following the withdrawal of $Ca^{+2}$. During this period of time, which corresponds to a single generation in the presence of $Ca^{+2}$, the static cells complete their current round of chromosome replication as judged by a 2-fold increase in content of DNA, a corresponding degree of resistance to irradiation with UV, and ability to resume growth in synchrony after a 90-minute lag following the reduction of temperature to 26° C. The specific activity of DNA polymerase in static cells is identical to that in dividing organisms. YANG et al. (1971b) showed that cells which remain static for 6 hours contain at least twice

the number of visible nuclei that can be observed within dividing cells. Accordingly, stasis with concomitant production of V and W reflects a block which occurs after the termination of chromosome replication but before the onset of invagination and division.

The reason for the appearance of V and W during stasis has not yet been determined and the role of these antigens in the process of infection is poorly understood. It seems unlikely that they would be expressed *in vivo* by organisms

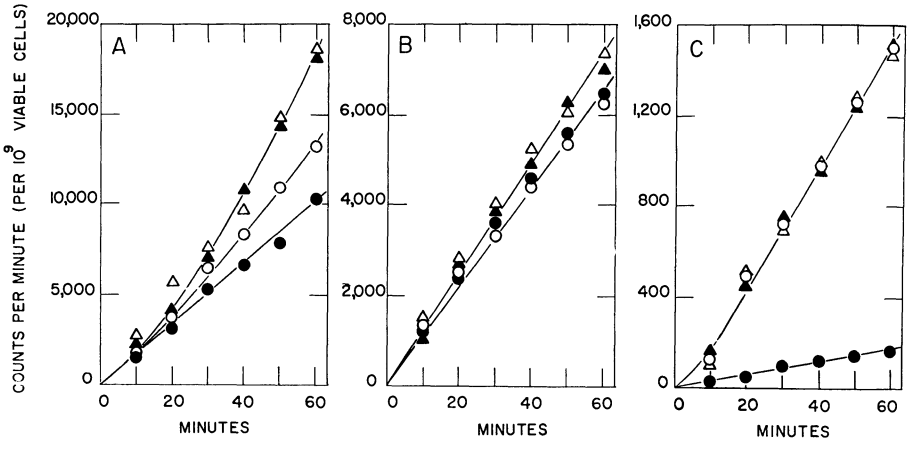


Fig. 3. Rates of synthesis of macromolecules at 37° C by cells of *Yersinia pestis* strain EV 75; *vwa*+ cells following stasis for 12 hr in the absence of added $Ca^{+2}$ (●), dividing *vwa*+ cells in the presence of $Ca^{+2}$ (○), dividing *vwa*− cells without $Ca^{+2}$ (▲), and dividing *vwa*− cells with $Ca^{+2}$ (△). A, incorporation of $^{14}$C-isoleucine into protein; B, incorporation of $^{14}$C-uracil into RNA; and C, conversion of $^{14}$C-uracil into thymidine triphosphate and subsequent incorporation into deoxyribonucleic acid. (After YANG and BRUBAKER, 1971a)

circulating within the vascular system because, as noted by HIGUCHI et al. (1959), blood contains sufficient $Ca^{+2}$ (0.0025 M) to promote growth (with repression of V and W). In contrast, V and W might be produced following phagocytosis because, according to KUGELMASS (1959), mammalian intracellular fluid contains 0.02 M $Mg^{+2}$ but no $Ca^{+2}$. In any event, *vwa*+ organisms are more immunogenic than *vwa*− mutants (BURROWS and BACON, 1958) and LAWTON et al. (1963) demonstrated that monospecific anti-V, but not anti-W, provides passive protection to mice against experimental plague.

## 2. Fraction 1 Antigen

Like the virulence antigens, the optimal temperature for production of the envelope or fraction 1 antigen is that of the mammalian host although small amounts are evidently present in a bound state during incubation at room remperature (FOX and HIGUCHI, 1958). Fraction 1 is also immunogenic in mice and guinea pigs (SCHUTZE, 1932; BAKER et al., 1952; CHEN, 1952; WALKER et al., 1952; SEAL, 1953; CHEN and MEYER, 1955; CRUMPTON and DAVIES, 1956; KEPPIE et al., 1958, 1960; LAWTON et al., 1960; EISLER et al., 1963a, 1963b;

BASOVA, 1966; BASOVA and FILIMONOVA, 1968), at least against *fra*$^+$ organisms (BURROWS and BACON, 1958; SURGALLA, 1960). The mutation to *fra*$^-$ may occur at high frequency as judged by the predominance of this cell type in mice which fail to survive challenge following immunization with *fra*$^+$ cells (BURROWS, 1957) and by rapid selection during continuous cultivation (PIRT et al., 1961). Genetic loss of ability to produce fraction 1 is without effect in the mouse (Table 1) but results in an approximate 1000-fold increase in $LD_{50}$ in the guinea pig (BURROWS, 1957). However, *fra*$^-$ cells remain fully infectious in the guinea pig via the intradermal route as evidenced by an $ID_{50}$ of 10 organisms determined on the basis of skin lesion, bubo development, and febrile reaction (DONOVAN et al., 1961).

BURROWS (1960, 1962) has applied the term F1$^\pm$ to mutants that fail to form visible envelopes at 37° C but retain the ability to release free fraction 1 antigen. Such strains resemble *fra*$^-$ organisms in being of reduced virulence in guinea pigs but not mice. The isolation of an F1$^\pm$ strain from a fatal case of human plague (WINTER et al., 1960) would indicate that the envelope does not determine virulence in man were it not for the possibility of F1$^\pm$ cells being selected *in vivo*. The mutation from *fra*$^+$ cells to *fra*$^-$ may reflect loss of one or more structural genes for fraction 1 whereas the less common mutation to the F1$^\pm$ phenotype could occur upon loss of a second gene or set of genes that regulate formation of the envelope (BURROWS, 1962). Mutation from *fra*$^-$ to *fra*$^+$ has not been reported. With the development of antiserum-agar plates which distinguish between *fra*$^+$ and *fra*$^-$ cells (ALBIZO and SURGALLA, 1968a), it should be possible to characterize the emergence of *fra*$^-$ mutants from *fra*$^+$ populations. Modifications of this medium were used to identify *Y. pestis* in grossly contaminated tissues of guinea pigs that had died of plague (ALBIZO and SURGALLA, 1968b). Fraction 1 antigen was determined by immunoelectrophoresis by DODIN and BRYGOO (1965).

### 3. Pesticin I

BEN-GURION and HERTMAN (1958) described a bacteriocin-like material produced by wild-type strains of *Y. pestis* which inhibits the growth of *Y. pseudotuberculosis*. This antibacterial activity, designated pesticin, was detected in supernatant fluids of 48 hour broth cultures but was not found in 24 hour cultures even after the oragnisms were disrupted by sonic vibrations. Pesticin was observed in 24 hour whole cultures, however, after irradiation with UV. Activity is destroyed by trypsin, heat, and extremes of pH and is 20 times more active when tested at 37° C than at 30° C. HERTMAN and BEN-GURION (1959). presented convincing evidence, based on demonstration of a requirement for amino acids and inhibition by chloramphenicol, that the expression of pesticin following irradiation represents *de novo* synthesis of protein.

The activity discovered by BEN-GURION and HERTMAN (1958) was designated pesticin I by BRUBAKER and SURGALLA (1961) upon the detection of a second antibacterial substance, termed pesticin II, which is produced by all tested

strains of *Y. pestis* and *Y. pseudotuberculosis* but is active against only a few *pst*$^-$ isolates of *Y. pestis*. High levels of $Ca^{+2}$ (~0.05 M) are necessary for the expression of maximum pesticin II activity which, like pesticin I, is destroyed by trypsin. Serotype I strains of *Y. pseudotuberculosis* (BURROWS and BACON, 1960; BRUBAKER and SURGALLA, 1961), certain strains of *E. coli*, including the colicin indicator strain $\phi$ (BRUBAKER and SURGALLA, 1961; SMITH and BURROWS, 1962), and a few isolates of *Y. enterocolitica* (*unpublished observations*) are sensitive to pesticin I. A nonpesticinogenic mutant of *Y. pestis*, isolated by HERTMAN and BEN-GURION (1959), is also sensitive to this substance, and LOGACHEV and TIMOFEEVA (1966) reported that isolates of *Y. pestis* which ferment rhamnose both produce and are sensitive to an activity that may be pesticin I as judged by its ability to kill cells of *Y. pseudotuberculosis*. BRUBAKER and SURGALLA (1961) noted that the antibacterial activity of pesticin I can be inhibited by hemin and $Fe^{+3}$ and this effect can in turn be reversed by $Ca^{+2}$, $Sr^{+2}$, or chelating agents. Inhibition of activity by $Fe^{+3}$ is mediated by a slowly diffusing anionic substance (pesticin I inhibitor) that is elaborated by both *pst*$^+$ and *pst*$^-$ organisms. When precautions are taken to eliminate $Fe^{+3}$ by use of ethylenediaminetetraacetate in excess $Ca^{+2}$, considerable pesticin I is detected in unirradiated cells (BRUBAKER and SURGALLA, 1962a). This observation, of course, does not preclude the possibility that additional activity might be detected following treatment with UV.

In a study of the mode of action of pesticin I, ELGAT and BEN-GURION (1969) showed that viable cells of *E. coli* strain $\phi$ decrease exponentially in broth in proportion to the concentration of added pesticin I. Similar reduction in viability of *Y. pseudotuberculosis* could only be obtained in the presence of added $Ca^{+2}$ (0.05 M); it would be of interest to see if this effect could be antagonized by $Fe^{+3}$. Although these workers stated that exposure to pesticin I results in arrest of DNA replication and degradation of RNA with little effect on production of protein, they used chemical methods of analysis which did not permit an estimation of synthetic rates. Nevertheless, total production of macromolecules in *E. coli* exposed to pesticin I resembles that in cells treated with colicin E2, a bacteriocin known to effect degradation of DNA (NOMURA and MAEDA, 1965). Furthermore, both pesticin I and colicin E 2 induce lysogenic *E. coli* strain $\phi$ (P1) but pesticin, unlike colicin E 2, is active in the presence of 2,4-dinitrophenol. Physical absorption of pesticin I to cells of sensitive *Y. pseudotuberculosis* was not detected.

Further work will be necessary to define with certainty the mode of action of pesticin I. At present, little is known about the expression of pesticin II and its effect on sensitive cells; in view of the ubiquitious distribution of its genetic determinant among yersiniae it seems probable that pesticin II is not a bacteriocin. Further attention to the effects of $Fe^{+3}$, hemin, and $Ca^{+2}$ on the activity of pesticin I should prove rewarding. BRUBAKER and SUMNER (*unpublished observations*) showed that the activity of a number of colicins is inhibited by hemin whereas that of colicin I and colicin V is enhanced by $Ca^{+2}$ and inhibited by $F^{+3}$.

DOMARADSKII et al. (1963) noted a correlation between production of the plague fibrinolytic-factor discovered by MADISON (1936) and the coagulase of JAWETZ and MEYER (1944). BRUBAKER et al. (1965b) subsequently showed that the ability to produce pesticin I is correlated with expression of coagulase and fibrinolysin which, according to BEESLEY et al. (1967), is associated with certain particulate fractions whereas pesticin I is soluble. The most obvious explanation for this correlation would be the existence of structural genes for pesticin I, coagulase, and fibrinolysin on an extrachromosomal replicon; mutational loss of this replicon would accordingly result in the concomitant loss of all 3 properties. Other possibilities exist such as mutational loss of a common activator, loss of an operator or regulator gene, or polarity effects but no evidence favoring these rather complex alternatives was obtained by BEESLEY et al. (1967). The production of coagulase and fibrinolysin by *Y. pestis* is therefore assumed to be a function of bacteriocinogenic conversion.

The relationship between pesticinogeny and virulence was defined by BRUBAKER et al. (1965a) who reexamined the coagulase-negative strains of EISLER (1961). As expected, all of these isolates lacked pesticin I and fibrinolysin but only one strain had retained all the then established determinants of virulence. Cells of this strain are of reduced virulence in mice by intraperitoneal injection and are avirulent via the subcutaneous route; the $LD_{50}$ following intravenous injection approximates that of wildtype cells which is low by all 3 routes of infection. This finding was interpreted to indicate that loss of the pesticin I determinant results in a decreased ability to invade the host due, presumably, to the concomitant loss of coagulase and fibrinolysin. The notion that fibrinolysin and coagulase rather than pesticin I *per se* are associated with virulence has not been proven with certainty by determining the $LD_{50}$ of strains possessing appropriate point mutations on the pesticin I determinant. BURROWS (1965) suggested that pesticin I may fulfill an independent role in enabling *Y. pestis* to obtain iron *in vivo*. The virulence of *pst*$^-$ cells is significantly reduced following intraperitoneal but not subcutaneous injection in mice which receive sufficient $Fe^{+2}$ by injection to saturate serum transferrin (BRUBAKER et al., 1965a). The effect of iron on experimental infections in mice is discussed in more detail in the next section.

According to DOMARADSKII (1966), the particulate fibrinolysin can be solubilized by extraction with urea, KSCN, or phenol indicating that this activity is protein in nature. IAROMIUK (quoted by DOMARADSKII, 1966) demonstrated that the fibrinolysin activates plasminogen and this finding was verified BEESLEY et al. (1967) who showed that activity is inhibited in the presence of $\varepsilon$-aminocaproic acid (0.03 M), a known inhibitor of urokinase, or by sufficient heat to destroy plasminogen. Lysis of fibrin is not dependent upon the presence of a host-specific proactivator thus the plague fibrinolysin resembles staphylokinase rather than streptokinase.

In order to demonstrate coagulase activity it is necessary to use large numbers of cells and a minimal concentration of anticoagulant (EISLER, 1961). Studies on the mode of action of coagulase have been hampered by the conco-

mitant occurrence of fibrinolysis; present evidence favors the hypothesis that the coagulase activates prothrombin rather than mimicking the action of thrombin (DOMARADSKII, 1966; BEESLEY et al., 1967). No direct evidence was obtained by BEESLEY et al., (1967) to show that the coagulase and fibrinolytic activities are functions of separate molecules, although their reactions are clearly not catalyzed by pesticin I. It should be noted that certain proteases can activate both plasminogen and prothrombin. Further work will be necessary to clarify this relationship.

## 4. Pigmentation

Using a defined solid medium containing galactose, salts, and 6 amino acids, JACKSON and BURROWS (1956a) showed that wild-type cells of *Y. pestis* absorb exogenous hemin and basic aromatic dyes and thus grow in the form of colored or pigmented colonies. In contrast, *pgm*$^-$ cells fail to absorb the pigments and grow as white colonies. Hemin is absorbed directly to the cell surface in an unaltered state and this process results in formation of large brittle masses of aggregated bacteria. Pigmentation is most pronounced at room temperature at pH 8.0 and is reduced or absent on media enriched with natural products. However, SURGALLA and BEESLEY (1969) showed that the dye congo red is strongly absorbed by *pgm*$^+$ cells when incorporated into common, commercially available laboratory media. Congo red agar did not permit significant pigmentation of isolates of *Salmonella, Shigella Klebsiella, Pseudomonas, Proteus, Bacillus,* or *Staphylococcus* although an intense reaction was observed with certain yeasts and members of the family *Micrococcaceae.*

SURGALLA et al. (1968) showed that a marked population-shift favoring *pgm*$^-$ cells occurs at 26° C in broth cultures during the death phase due to accelerated loss of *pgm*$^+$ organisms. This loss could be eliminated by reducing the terminal pH to neutrality with HCl or by maintaining electrolyte balance with $Na^+$ rather than $K^+$. These findings did not distinguish between selection of *pgm*$^-$ cells and apparent loss of *pgm*$^+$ organisms due to clumping. SURGALLA (1960) earlier noted a correlation between ability to express the pigmentation reaction and production of a substance in aerated broth at 26° C which promotes clumping and adherence of cells to the sides of the flask. In this context it should be mentioned that a similar effect observed during storage can be minimized by treatment of the cells with DNAse (WESSMAN and MILLER, 1966); BEKKER and KUTSEMAKINA (1960) noted that NaCl-extractable DNA was excreted by *Y. pestis* during growth on solid medium.

The nature of the surface component that promotes the pigmentation reaction has not been determined with certainty. This structure might be lost or become covered by another surface layer upon mutation to *pgm*$^-$. One clue which may lead to a better understanding of pigmentation was the finding that *pst*$^-$, *pgm*$^+$ cells of *Y. pestis,* but not *pst*$^+$, *pgm*$^+$ or *pst*$^-$, *pgm*$^-$ organisms, are sensitive to the antibacterial action of pesticin I (BRUBAKER, 1969). In these experiments it was assumed that the *pst*$^+$, *pgm*$^+$ cells were immune to exogenous

pesticin I and that organisms of the *pst*$^-$, *pgm*$^-$ genotype were resistant by virtue of mutational loss of absorption sites. This relationship was exploited by plating *pst*$^-$, *pgm*$^+$ cells on agar containing pesticin I in order to quantitatively recover *pgm*$^-$ mutants; a mutation rate from *pgm*$^+$ to *pgm*$^-$ of $10^{-5}$ was determined by this procedure. It is important to note, however, that at least on *pst*$^-$, *pgm*$^+$ strain is not sensitive to pesticin I, indicating that the pigmentation and pesticin I absorption site are not necessarily identical. The mutation to *pgm*$^-$ seems to be irreversible.

JACKSON and BURROWS (1956b) made the important observation that cells that are avirulent due to mutation to *pgm*$^-$ can be restored to full virulence in mice by concomitant injection of sufficient $Fe^{+2}$ to saturate serum transferrin and thus provide an excess of iron in the plasma. This observation suggested that *pgm*$^+$ cells are normally capable of containing iron *in vivo* from some source that is not available to *pgm*$^-$ mutants. However, JACKSON and MORRIS (1961) were unable to demonstrate a selective ability of *pgm*$^+$ cells to multiply in mouse or human serum although growth of both *pgm*$^+$ and *pgm*$^-$ organisms is dramatically increased in such sera by addition of $Fe^{+2}$ or $Fe^{+3}$ but not by hemin or lysed erythrocytes. Accordingly, if *pgm*$^+$ cells do selectively obtain iron *in vivo*, the source is not a normal component of serum. The restriction against *pgm*$^-$ cells which is relieved in mice by injected iron is also imposed upon *pgm*$^+$ organisms, as judged by the dramatically rapid infection that occurs following the concomitant administration of $Fe^{+2}$ and wild-type *Y. pestis* (*unpublished observations*). Attempts by JACKSON and MORRIS (1961) to induce the growth of *pgm*$^+$ and *pgm*$^-$ cells in human serum by addition of $Mn^{+2}$, $Co^{+2}$, $Ni^{+2}$, $Cu^{+2}$, $Zn^{+2}$, $Mg^{+2}$, or $Ca^{+2}$ were not successful. As noted previously, the virulence of *pst*$^-$ cells that retained the remaining determinants of virulence can also be enhanced in mice receiving $Fe^{+2}$ by injection (BRUBAKER et al., 1965a); cortisone, like iron, promotes lethal infections of *pgm*$^-$ cells in mice (PAYNE et al., 1955; YUSCHENKO et al., 1960). FRIEDBERG and SHILO (1965) reported that the virulence of *pgm*$^-$ cells can be enhanced in mice by injection of certain neutral and acidic polysaccharides of high molecular weight, but these effects may again be on the host rather than on the bacteria.

JACKSON and MORRIS (1961) showed that cells of *S. typhimurium* and *Y. pseudotuberculosis* grow in serum provided that iron is present; the latter organism, in fact, is rapidly killed in the absence of added iron. Like *pgm*$^-$ or *pst*$^-$ cells of *Y. pestis*, the lethality of *Y. pseudotuberculosis* in mice is enhanced by concomitant administration of $Fe^{+2}$ (BURROWS and BACON, 1960) or cortisone (YUSHCHENKO et al., 1960) and injected $Fe^{+2}$ also favors the virulence of *Listeria monocytogenes* (SWORD, 1966), certain enteric bacteria (MARTIN et al., 1963; BULLEN and ROGERS, 1969), and *P. septica* (BULLEN et al., 1968; BULLEN and ROGERS, 1969). In fact, BULLEN et al. (1967) made the significant discovery that the protection normally provided against *Clostridium welchii* type A by specific antiserum is abolished by injection of iron prior to infection. Identical results were subsequently obtained with *P. septica* (BULLEN et al., 1968). These workers also demonstrated that cells of *E. coli* strain 0111 are killed in normal

rabbit serum unless iron is added or the serum is inactivated by heat. These findings suggest that a component of complement or some antibacterial factor may be inactivated by iron; an antirespiratory *7S* globulin of this description has been described (BORNSIDE et al., 1964). The injection of $F^{+2}$ into guinea pigs does not result in increased sensitivity to *pgm*$^-$ mutants of *Y. pestis* (JACKSON and BURROWS, 1956b). It is evident from these experiments that the hypothesis that *pgm*$^+$ cells of *Y. pestis* possess some unique method for obtaining iron *in vivo* (BURROWS, 1963; BURROWS, 1965) may be overly simplistic. Any alternative, however, may have to await isolation of a relevant antibacterial principle in normal serum, and determination of the mechanism of its neutralization by iron.

## 5. Purine Independence

BACON et al. (1951) first showed that a mutational loss of the ability to synthesize purines *de novo* results in avirulence of *S. typhosa* and this observation was later extended to *Klebsiella pneumoniae* (GARBER et al., 1952), *S. typhimurium* (GOWEN et al., 1953; FURNESS and ROWLEY, 1956), *Y. pestis* (BURROWS, 1955), *Pseudomonas pseudomallei* (LEVINE and MAURER, 1958), and *Bacillus anthracis* (IVÁNOVICS and MARJAI, 1964; IVÁNOVICS et al., 1968). BURROWS (1955) did not identify the nature of the metabolic block in his purine-auxotrophs of *Y. pestis* which, in the case of one strain said to possess all remaining determinants of virulence, exhibited an $LD_{50}$ of $<10^8$ and $<10^{10}$ cells in mice and guinea pigs, respectively. Purine auxotrophs of other gram-negative bacteria retain considerable virulence when the metabolic block occurs prior to the formation of IMP. Gross loss of virulence is only noted in mutants blocked between IMP and AMP or between IMP and GMP and only the former mutation results in avirulence in the case of *B. anthracis*. Purine-dependence in *Y. pestis* was reinvestigated by BRUBAKER (1970) who found that blocks in the *de novo* synthesis of IMP effects only slight reduction of virulence in mice ($LD_{50} \sim 10^2$ cells) whereas loss of guanosine monophosphate synthetase (*gua A*) results in a comparable value of $>10^8$ cells. Mutants blocked between IMP and AMP which retained all of the remaining determinants of virulence were not isolated during this study, thus the avirulence of adenine auxotrophs has not been established with certainty. An assumed inability of purine auxotrophs to obtain adenine or guanine *in vivo* is generally believed to account for avirulence.

As noted previously, *Y. pestis* lacks detectable adenine deaminase and thus must rely on the histidine cycle for conversion of exogenous adenine to IMP. Another interesting aspect of purine metabolism was the finding that *pgm*$^+$ cells, under conditions that support the pigmentation reaction (incubation at 26° C a minimal medium), are unable to convert exogenous guanine to IMP whereas this conversion is readily performed by *pgm*$^-$ mutants or by *pgm*$^+$ cells cultivated under conditions that do not support the pigmentation reaction (incubation at 37° C or at 26° C in a highly enriched medium). This phenotypic

inability to form IMP from exogenous guanine is associated with absorption of the latter to the cell surface in a manner similar to that observed with hemin, basic dyes, congo red, and pesticin I (BRUBAKER, 1970).

## C. Potential Determinants of Virulence

Proof that the 5 factors discussed in the previous section are associated with the expression of virulence was obtained by correlating an increase in $LD_{50}$ with their individual mutational loss. The purpose of this section is to review certain properties that may be involved in the pathogenic process but which are intimately associated with the structural or metabolic integrity of the cell; mutational loss of these determinants would presumably be lethal. Also included are potential determinants of virulence which do undergo rare mutational change as judged by their absence in certain strains which, unfortunately, are already rendered avirulent by loss of one or more of the recognized factors previously described.

### 1. Murine Toxin

Of unknown significance in the pathology of plague is the soluble, heat labile, formalin-sensitive, toxic protein first described by RAMON et al. (1947). Environmental factors have not been reported to influence the production of this toxin which was prepared by differential precipitation with $(NH_4)_2SO_4$ (BAKER et al., 1952) and by various absorption and precipitation procedures (AJL et al., 1955) and by curtain electrophoresis (AJL et al., 1958a; APIVACK and KARLER, 1958). This type of molecule, which is found within the cytoplasm, was termed toxin B by MONTIE et al. (1964) upon identification of a second toxic molecule, designated toxin A, which is associated with the cytoplasmic membrane. As judged by Sephadex filtration, the molecular weights of toxins A and B are 240,000 and 120,000, respectively (MONTIE et al., 1966) and both proteins dissociate in sodium dodecyl sulfate to yield subunits with molecular weights of 10,000 to 12,000. These subunits retain about 60% of the toxicity of the parent molecules (MONTIE et al., 1968). Nontoxic subunits of higher molecular weight were obtained by other methods and both toxins can be resolved electrophoretically in a phenol-acetic acid-water system to yield 2 components apiece; the major polypeptide is common to both toxins whereas the minor polypeptides are chemically distinct (MONTIE and MONTIE, 1969).

Although these toxins are not effective in the guinea pig, rabbit, dog, or monkey (SCHÄR and MEYER, 1956), they are highly toxic for rats and mice. The $LD_{50}$ of the preparation of AJL et al. (1958a) was less than one $\mu$g in the latter species, thus the soluble plague toxin is generally termed "murine" toxin. The oxidation of $\alpha$-keto acids by cell-free microbial extracts and crude tissue homogenates can be inhibited by murine toxin and this inhibition can be reversed by excess $NAD^+$ but not $NADP^+$ (AJL et al., 1958b). Subsequent study showed that the exogenous respiration of rat heart mitochondria is inhibited by murine toxin whereas that of heart mitochondria from toxin-

resistant animals is not affected; no such correlation was obtained with mitochondria from other organs (PACKER et al., 1959; RUST et al., 1963; KADIS et al., 1963). However, the respiration of chemically and physically disrupted rabbit heart mitochondria is significantly inhibited by murine toxin whereas heart mitochondria obtained from sensitive but immunized animals are not affected (KADIS et al., 1963). In all cases where antirespiratory activity can be noted, the toxin causes mitochondrial swelling which can be prevented by respiratory inhibitors and reversed by ATP and $Mg^{+2}$ (KADIS and AJL, 1963). Murine toxin does not influence the respiratory chain between cytochrome *c* and oxygen as judged by inability to effect the oxidation of ascorbate by rat heart or liver mitochondria in the presence of tetramethylphenylenediamine. Similarly, no effect is observed on the specific activity of $NADH_2$-dehydrogenase, the first enzyme of the respiratory pathway; however, both purified and mitochondrial $NADH_2$-cytochrome *c* reductase and reduced coenzyme Q-cytochrome *c* reductase activities are inhibited indicating that murine toxin acts by preventing the reduction of coenzyme Q (KADIS et al., 1965; KADIS et al., 1966).

Some alternative proposals regarding the mechanism of action of murine toxin have been reviewed by DOMARADSKII (1966) and WALKER (1967) who emphasize the role of lipopolysaccharide in the intoxication of large animals. VASIL'EVA (1967) has detected an effect of murine toxin on pyruvate oxidase and lactate dehydrogenase systems in plague-sensitive animals and DZHAPARIDZE et al. (1967) showed a decrease of malate and fumarate in the heart and liver of intoxicated mice and rats. HILDEBRAND et al. (1966) investigated the mechanism of circulatory failure induced in rats by murine toxin. However, significant electrocardiographic changes, typical of those caused by murine toxin, are not always obtained in the resistant monkey (HOESSLY et al., 1955) or sensitive rat (RUST et al., 1963) during infection with live cells. These findings, plus the inability of earlier investigators to obtain typical endotoxin from *Y. pestis*, have resulted in attempts to isolate toxins produced only *in vivo* (KEPPIE et al., 1957; H. SMITH et al., 1960) or to show synergism between murine toxin and lipopolysaccharide (COCKING et al., 1960; WALKER, 1967). These studies have been without marked success and intoxication can generally be attributed to murine toxin or to the lipopolysaccharide described below. The ability to produce murine toxin can be lost by mutation and it would be of interest to see if such mutants still produce the nonidentical polypeptides of toxins A and B described by MONTIE and MONTIE (1969). An atoxic mutant which retains the recognized determinants of virulence is needed to define the importance of murine toxin in experimental plague.

## 2. Endotoxin

The lipopolysaccharide first isolated from *Y. pestis* with hot phenol by the method of WESTPHAL and LÜDERITZ (1954) is considerably less toxic than similar endotoxins obtained from other gram negative bacteria (DAVIES, 1956).

Preparation of this type, however, produces symptoms and pathological changes typical of endotoxic shock (COCKING et al., 1960; LARRABEE et al., 1965; WALKER et al., 1966). Employing a modification of the cold phenol procedure of TAUBER and RUSSELL (1961), preparations of lipopolysaccharide were obtained from *Y. pestis* by ALBIZO and SURGALLA (1970) at a yield of approximately 1 % which represents a ratio of about 1 mg of product per $1.8 \times 10^{11}$ cells. The $LD_{50}$ of this type of preparation is about 500 μg in guinea pigs and mice and only 32 μg in rabbits. Concentrations of endotoxin approaching 500 μg would therefore be present in $10^{11}$ cells which approximates the number reported by COCKING et al. (1960) to be present in guinea pigs dying of plague. This figure is considerably more than the minimum number of bacteria estimated by WALKER (1967) to exist in moribund mice. Accordingly, plague endotoxin alone could theoretically account for death in guinea pigs whereas murine toxin might be expected to contribute to lethality in mice. The lipopolysaccharide of ALBIZO and SURGALLA (1970) can also evoke a biphase pyrogenic response in rabbits, induce tolerance in mice to endotoxin, stimulate rapid resistance to nonspecific infection, and produce the localized and generalized Swartzman reaction in rabbits.

### 3. Metabolic Factors

It is obvious that the mammalian host cannot indefinitely support a geometrically increasing population of parasites. The only question in such a situation, which seems to occur in the terminal stages of experimental plague, is whether death is an effect of toxic large molecules or a result of depletion of the host pools with concomitant accumulation of metabolic waste products. The release of $NH_3$, which in itself is toxic to mammals, during the catabolism of host amino acids might contribute to lethality although convincing evidence to this effect has not yet appeared. An interesting possibility is that the peculiar ability of the yersiniae to oxidize glutamate with release of α-ketoglutarate and $NH_3$ would permit the host to regenerate glutamate (via glutamate dehydrogenase) which would again be parasitized. Considerable energy could therefore be obtained by the bacteria (in the form of reduced pyridine nucleotides) from a cyclic pathway of this sort. Again, the relationship between this type of catabolic process and pathogenicity has not been defined; further study of amino acid turnover *in vivo* might result in a clearer understanding of biochemical events associated with mortality.

### 4. Enzymes

Early reports in the literature stated that *Y. pestis* produces a hemolysin and hyaluronidase. The former is now attributed to the excretion of long-chain fatty acids and attempts to isolate the latter have not been successful; production of other invasins such as DNAse, lecithinase, and protease was not verified (DOMARADSKII, 1966). WOODWARD (1944) reported that an RNAse activity is located on the cell-surface of *Y. pestis;* attempts to correlate this enzyme with expression of a recognized determinant of virulence have not been reported.

ROCKENMACHER (1949) stated that avirulence is associated with production of levels of catalase. This observation was not verified by BURROWS et al. (1964) who found that virulent strains and avirulent mutants of various genotype all produce extremely high levels of catalase; similar findings were reviewed by DOMARADSKII (1966). BRUBAKER and BEESLEY (*unpublished observations*) have noted that certain strains of *Y. pseudotuberculosis* and *pst*$^-$ mutants of *Y. pestis* can directly convert fibrinogen to fibrin. This reaction may occur via a "clumping factor" analogous to that produced by staphylococci. The activity of such an enzyme would be masked in *pst*$^+$ cells by fibrinolysin.

As stated previously, enzymes associated with the *de novo* biosynthesis of purines are essential for maximum virulence. Pyrimidine, vitamin, or amino acid auxotrophs of full virulence have been isolated indicating that the corresponding anabolic enzymes are not required for growth *in vivo* (BURROWS, 1963; *unpublished observations*). However, an isolate possessing all established determinants of virulence but blocked between L-citrulline and L-arginine, is of reduced virulence in guinea pigs (BRUBAKER and BEESLEY, *unpublished observations*); this finding is unexpected in view of the frequent isolation of arginine-dependent strains from nature (BURROWS and GILLETT, 1966). Little is known about the virulence of mutants blocked in carbohydrate or amino acid catabolism.

## 5. Antigens

The major antigens 3, 4, and 5 of CRUMPTON and DAVIES (1956) are produced during growth *in vitro* at 37° C but not at room temperature. Antigen 3 corresponds to fraction 1 and it is now established that antigens 4 and 5 are identical to antigens I and E of LAWTON et al. (1960). The fact that the temperature-dependent V, W, and fraction 1 antigens are associated with pathogenicity suggests that antigens 4 and 5 might also be determinants of virulence. Although no role has been determined for antigen 5, the ability to produce antigen 4 in conjugation with fraction 1 is correlated with smooth colony morphology and stability in suspension. This antigen was purified by CRUMPTON and DAVIES (1957) and shown to be a protein; subsequent study by PIRT et al. (1961) demonstrated that optimal synthesis of antigen 4 occurs at pH 5.9 with no production occurring at pH 6.9 or above. According to BURROWS (1963), antigen 4 is probably identical to the "pH 6 antigen" of BEN-EFRAIM et al. (1961) which imparts a reduction in electrophoretic mobility to the bacterial cell, agglutinates erythrocytes, induces primary inflammatory reactions of the skin, and is cytotoxic (BICHOWSKI-SLOMNICKI and BEN-EFRAIM, 1963). Mutants lacking the ability to produce antigen 4 have been noted; such isolates have either been *vwa*$^-$ or *pgm*$^-$ and are thus unsuitable for determination of virulence.

PETTENKOFER and BICKERICH (1960) reported that cells of *Y. pestis* strongly absorbed anti-H lectin of *Laburnum alpinum* and thus share an antigen similar to human blood group H substance. This observation raised the possibility that the low incidence of group O in populations of the ancient plague centers

occurred via selection of ability to produce anti-H (VOGEL et al., 1960). However, the experimental findings that led to this interpretation have been seriously questioned by SPRINGER and WIENER (1962).

## III. Yersinia pseudotuberculosis

### A. Physiology

Wild-type cells of *Y. pseudotuberculosis* grow at room temperature in media containing salts and a fermentable carbohydrate although a few isolates require added pantothenate or thiamin; the addition of glutamate, thiamin, and pathothenate generally favor growth at 37° C (BURROWS and GILLETT, (1966). *Y. pseudotuberculosis*, unlike *Y. pestis*, can utilize L-methionine as a sole source of sulfur (*unpublished observations*). The diagnostic characteristics of *Y. pseudotuberculosis* have been reviewed by MOLLARET (1962) and those properties that serve to distinguish this species from *Y. pestis* are discussed in section V. Unlike *Y. pestis*, the growth of *Y. pseudotuberculosis* is generally more rapid at 37° C than at room temperature; generation times of approximately 90 min have been noted in the medium of HIGUCHI et al. (1959) by YANG and BRUBAKER (1971 a). Growth of *Y. pseudotuberculosis* can be initiated at 37° C in this medium with smaller inocula than that required for *Y. pestis*, but the former is nevertheless sensitive to high concentrations of free amino acids (BRUBAKER, 1967).

*Y. pseudotuberculosis* exhibits operational Embden-Meyerhof and hexose monophosphate pathways; the Entner-Doudoroff pathway functions during the catabolism of gluconate (BRUBAKER, 1968). The organisms are assumed to possess a complete tricarboxylic acid cycle but the existence of this mechanism has not been formally proven. As judged by studies with an amino acid analyzer, exogenous L-serine and L-aspartate are very rapidly destroyed during growth at 37° C in an enriched medium. Following a short adaptive lag, L-glutamate disappears at an equal rate; the destruction of L-proline, L-threonine, and glycine occurs more slowly and the remaining naturally occurring amino acids do not appear to be catabolized, at least during the logarithmic phase of growth (BRUBAKER, 1967). Under certain conditions, the oxidation of glutamic acid by *Y. pseudotuberculosis* may result in nearly stoichiometric yields of α-ketoglutarate and $NH_3$ (*unpublished observations*). No other unusual properties involving catabolism in *Y. pseudotuberculosis* have been reported. The central anabolic pathways in this species are presumably identical to those in other enteric bacteria.

Cells of *Y. pseudotuberculosis* may be somewhat larger than those of *Y. pestis* but such differences are not consistently observed. However, cells of the former are generally motile with parapolar or peritrichous flagella during growth at room temperature but not at 37° C. Fraction 1 antigen is not produced by *Y. pseudotuberculosis* but a cross reaction with sera containing antibodies against the plague capsule has been reported (THAL, 1956). Lipopolysaccharides

from *Y. pseudotuberculosis* have been studied in detail and exhibit typical endotoxin activity. Of interest is the finding that O-antigen specificity in *Y. pseudotuberculosis* is correlated with the production of 3,6-dideoxyhexoses. Thus the serotypes I, II, III, IV, and V of THAL (1954) are known to contain paratose, abequose, paratose (in different linkage), tyvelose, and ascarylose respectively (DAVIES, 1958; 1961). The presence of abequose and tyvelose explains the cross reactions described by KNAPP (1965) with salmonellae of groups B and D (WESTPHAL et al., 1960). Serotypes I, II, IV, and V have recently been divided into subgroups A and B and a new serotype VI has been described (THAL and KNAPP, 1971); the corresponding chemotypes have not been reported. The biosynthesis of these uncommon sugars by *Y. pseudotuberculosis*, which does not incorporate rhamnose into lipopolysaccharide, has received intensive study (TINELLI et al., 1963; MATSUHASHI and STROMINGER, 1967). As noted previously, only serotype I strains of *Y. pseudotuberculosis* are sensitive to pesticin I, thus paratose may be associated with the absorption of this substance. No information is available regarding the structure and composition of the cytoplasmic membrane or lipid content of *Y. pseudotuberculosis*.

## B. Virulence

Freshly isolated strains of *Y. pseudotuberculosis* are *vwa*$^+$ (BURROWS and BACON, 1960) but fail to produce fraction 1, pesticin I, and murine toxin. Wild-type cells were originally classified as *pgm*$^+$ (BURROWS, 1963) but the intensity of pigmentation never matched that observed with wild-type *Y. pestis* (SURGALLA and BEESLEY, 1969). Somewhat lighter colored colonies of *Y. pseudotuberculosis* have been obtained by the selective procedure of SURGALLA et al. (1968) but cells of this phenotype proved to be unstable. Accordingly, wild-type *Y. pseudotuberculosis* is known with certainty to share only 2 of the 5 determinants of virulence expressed by *Y. pestis*, namely, the abilities to produce virulence antigens and to synthesize purines.

The $LD_{50}$ determined by the subcutaneous and intraperitoneal routes in mice with one *vwa*$^+$, *pur*$^+$ strain of *Y. pseudotuberculosis* is about $10^4$ cells whereas that obtained by intravenous infection is 39 cells. In contrast, an $LD_{50}$ of about 200 cells has been recorded by the subcutaneous and intraperitoneal routes in mice receiving a concomitant injection of sufficient $Fe^{+2}$ to saturate serum transferrin (BRUBAKER et al., 1965a). Cells of the *vwa*$^-$, *pur*$^+$ genotype have proved to be completely avirulent in normal or irontreated mice by all 3 routes of injection ($LD_{50} > 10^7$ cells). A completely different response has been obtained in guinea pigs where the $LD_{50}$ by intravenous and intraperitoneal injection was $>10$ with *vwa*$^+$, *pur*$^+$ cells and about 50 with *vwa*$^-$, *pur*$^+$ cells. Approximately identical values have been recorded following subcutaneous injection but the latter determinations were complicated by a high incidence of chronic disease with individual animals surviving for periods of 1 to 2 months (BRUBAKER, *unpublished observations*). Similar results obtained by YUSCHENKO (1967) can be interpreted within this framework. Accordingly, the ability of

*Y. pseudotuberculosis* to produce V and W is essential for the expression of virulence in mice but not guinea pigs. BURROWS (1963) found that purine auxotrophs of *Y. pseudotuberculosis* are avirulent and this observation was verified to the extent of showing that the $LD_{50}$ of a *vwa*$^-$ mutant blocked prior to the formation of IMP is about $10^4$ cells in guinea pigs. The corresponding value of a *vwa*$^-$ mutant blocked between IMP and AMP (*ade B*) is $>10^8$ cells (*unpublished observations*).

Cells of *Y. pseudotuberculosis* differ somewhat from *Y. pestis* with regard to the expression of V and W. The mutation of *vwa*$^-$ in the former is correlated with an alteration to rough colonial morphology which is not observed in the case of *Y. pestis* (BURROWS and BACON, 1960). The nature of this change has not been defined; presumably it does not reflect an alteration of O-antigen structure. A few *vwa*$^+$ but rough isolates of *Y. pseudotuberculosis* have been isolated (*unpublished observations*). Virulent strains of *Y. pseudotuberculosis*, like those of *Y. pestis*, exhibit a nutritional requirement for $Ca^{+2}$ (BRUBAKER, 1967). Of interest, however, is the fact that withdrawal of $Ca^{+2}$ from cells of *Y. pseudotuberculosis* at 37° C does not result in immediate cessation of growth as is the case with *Y. pestis*, but 2 or 3 divisions are completed prior to the onset of stasis (YANG and BRUBAKER, 1971a). Static cells of *Y. pseudotuberculosis* are sensitive to high concentrations of $Na^+$ yet this cation favors growth in the presence of $Ca^{+2}$. Static cells of this species may undergo auto-agglutination (BRUBAKER, 1967). The mutation rate to *vwa*$^-$ in *Y. pseudotuberculosis*, like that in *Y. pestis*, is $10^{-4}$ (*unpublished observations*).

A number of potential determinants of virulence are shared with *Y. pestis* including antigens 4 and 5. Serotype III isolates may produce an exotoxin which is distinct from murine toxin (SCHÄR and THAL, 1955). The pseudotuberculosis toxin has been partially purified and further characterized by BROWN et al. (1969).

## IV. Pathogenesis

### A. Plague

*Post mortem* changes in experimental plague are dependent upon the route of injection and vary somewhat from one host to another. In guinea pigs, necrosis and edema is noted at both the site of injection and at the regional lymph glands which become swollen (bubos). The spleen is enlarged and this organ as well as the liver and lungs may exhibit necrotic nodules containing viable bacteria. A similar pattern exists in mice and rats where the spleen becomes darkened upon enlargement and the formation of a pleural exudate is common; septicemia is generally observed during the terminal stages of the disease (WILSON and MILES, 1964). The symptoms and pathology of plague in monkeys was described by MCCRUMB et al. (1953) and SPECK and WOLOCHOW (1957); the pneumonic form of the disease was further discussed by MEYER, (1961).

Studies of early changes following infection have led to a correlation between virulence and resistance to phagocytosis which, according to MEYER (1950) and ENGLESBERG et al. (1954), is mediated by fraction 1 antigen. Virulence has also been equated with resistance to phagocytosis by BURROWS and BACON (1956) who have attributed the latter to the activity of V and W antigens. These workers stated that both *vwa*$^+$ and *vwa*$^-$ cells are sensitive to ingestion by mouse polymorphonuclear leukocytes following growth *in vitro* at 28° C, but that subsequent incubation *in vivo* or *in vitro* for 3 hours at 37° C results in *vwa*$^+$ but not *vwa*$^-$ cells becoming resistant to phagocytosis. Incubation at 37° C or *in vivo* for 3 hours permits expression of the virulence antigens but does not result in formation of a morphologically distinct envelope. However, when the organisms are grown *in vivo* or *in vitro* at 37° C for 9 to 16 hours, a uniformly high resistance to phagocytosis is observed and this change has been correlated with the formation of visible envelopes.

These observations were supported by the finding that virulent cells grown at 26 C are rapidly cleared from lungs of guinea pigs (FUKUI et al., 1957a) and mice (SMITH et al., 1957) following infection by aerosol; those organisms that survive for 6 hours in guinea pigs and 16 hours in mice were found to be able to initiate logarithmic growth. The initial reduction in viability is associated with a high rate of phagocytosis and this observation was correlated by FUKUI et al. (1957a) with the observation that *vwa*$^+$ cells that had been cultivated *in vivo* or *in vitro* at 37° C in an enriched medium are neither phagocytized nor cleared. Cells of a genetically unrelated avirulent strain, now known to be *vwa*$^-$, *pst*$^-$, and unable to produce antigen 4, can never initiate rapid growth in guinea pig lungs.

These findings suggested that V and W are indeed able to confer resistance to phagocytosis. However, JANSSEN et al. (1958) subsequently showed that virulent phagocytosis-sensitive cells, cultivated *in vitro* at 26° C, and virulent phagocytosis-resistant cells, cultivated *in vivo*, are rapidly removed from the vascular system of guinea pigs by fixed macrophages of liver and spleen. Accordingly, the ability to resist ingestion by circulating phagocytes has no effect on early survival in blood although the progeny of initially phagocytosis-resistant cells subsequently were found to appear in blood and organs about 10 hours earlier than do those of initially phagocytosis-sensitive cells. These experiments did not distinguish between the possibility that phagocytosis-resistant cells can survive and multiply within fixed macrophages or that such cells can multiply at a rate that exceeds their capacity to be destroyed by the reticuloendothelial system. JANSSEN et al. (1963) verified that *vwa*$^+$, *fra*$^+$ cells become highly resistant to phagocytosis by neutrophiles and free macrophages following cultivation *in vivo*. However, these workers also showed that *vwa*$^-$, *fra*$^+$ cells become equally resistant to ingestion after similar cultivation whereas *vwa*$^+$, *fra*$^-$ organisms remain sensitive to phagocytosis. The results of this study, which are in conflict with the hypothesis that V and W inhibit phagocytosis, were interpreted to suggest that virulence is a function of intracellular survival rather than resistance to phagocytosis.

BURROWS and BACON (1956) have stated that phagocytosis-sensitive cells of *Y. pestis* are killed following ingestion by mouse polymorphonuclear leukocytes. The same conclusion was drawn by CAVANAUGH and RANDALL (1959) who made the important discovery that cells ingested by free macrophages undergo rapid intracellular growth. According to KENIG et al. (1968), organisms ingested by guinea pig neutrophiles are destroyed but cell walls and cytoplasmic membranes remain visible for 5 days. However, JANSSEN and SURGALLA (1969) later showed that *Y. pestis* can survive for at least 42 hours in neutrophiles as judged by direct observation of intracellular bacteria following incubation of exudates for 24 hr at 23° C. Similar results have been obtained with macrophages which often become infected by ingesting and digesting infected neutrophiles. These workers have attributed earlier difficulties in detecting intracellular bacteria to their existence in some unrecognized morphologic state or to occlusion by nuclei or organelles within the phagocyte. No differences in intracellular survival or growth have been detected between *vwa*$^+$ and *vwa*$^-$ cells. In a related study, the effect of *Y. pestis* in inhibiting mitosis of human embryo cells has been studied by KHESIN et al. (1966).

In summary, it has been established that cells cultivated at room temperature are susceptible to ingestion by free phagocytes but that the organisms are able to survive intracellularly. Growth *in vivo* or at 37° C prior to infection results in phenotypic resistance to ingestion by free phagocytes and this property is correlated with expression of fraction 1 antigen but not V and W. Both phagocytosis-sensitive and phagocytosis-resistant organisms are rapidly cleared by fixed macrophages of the reticuloendothelial system but the progeny of the latter appear sooner in blood and tissues. These observations have prompted JANSSEN et al. (1958) and JANSSEN and SURGALLA (1969) to suggest that virulence is related to an ability to supress the proliferation of or neutralize the antibacterial activity of the reticuloendothelial system.

The role, if any, of V and W in this process remains obscure. As noted previously, the production of these antigens *in vitro* is inhibited by $Ca^{+2}$ which is distributed at high concentration in plasma and interstitial spaces but does not exist in ionized form within phagocytes. Accordingly, one might expect that V and W would be produced intracellularly; such production could conceivably inhibit the capacity of the reticuloendothelial system to proliferate or to destroy ingested organisms. There is, however, no evidence at present which demonstrates that biochemical processes of the host are directly influenced by the virulence antigens. It seems equally likely that V and W could modify the metabolic response of the parasite (*see* section II. B. 1) in order to ensure intracellular survival. Stimulation of the reticuloendothelial system by lipopolysaccharide prior to infection results in a significant increase in $LD_{50}$ (LAWTON and SURGALLA, 1963; ALBIZO and SURGALLA, 1970) and it would be interesting to determine if host changes associated with such stimulation are prevented by the presence of virulent cells.

The ability to pigment is generally assumed to be associated with extracellular survival but the relationship between this determinant and iron, serum

transferrin, and antibacterial principals of serum remains to be defined (*see* section II. B. 4). The possibility exists, of course, that the pigmentation reaction also influences the response of the reticuloendothelial system. Pesticinogeny is undoubtedly correlated with invasiveness via fibrinolysin and coagulase and these activities may also account for many of the gross pathological changes noted earlier in this section (*see* DOMARADSKII, 1966). As already discussed, the expression of fraction 1 promotes resistance to ingestion by free phagocytic cells but such resistance only slightly modifies the course of disease.

## B. Pseudotuberculosis

As noted previously (section III. B.), *vwa*$^-$ cells of *Y. pseudotuberculosis* are avirulent in mice but are highly virulent in guinea pigs. The pathological changes that occur following infection in mice closely resemble those caused by *Y. pestis* except that deposits of fibrin are sometimes observed in the peritoneal cavity. Chronic disease is not observed in mice and seldom occurs in guinea pigs following intravenous or intraperitoneal injection. *Post mortem* changes associated with acute disease in guinea pigs also resemble those caused by *Y. pestis* although deposits of fibrin may again be observed. The sub-acute and chronic forms of pseudotuberculosis in guinea pigs result in emaciation which becomes progressively severe. Local and regional lymph glands become enlarged and caseous and numerous grey nodules are observed in the liver, spleen, and lungs (WILSON and MILES, 1964). The host-range of *Y. pseudotuberculosis* extends to birds, various predatory animals, and primates (MEYER, 1965); both acute and chronic forms of disease have been reported in man, the latter commonly taking the form of a mesenteric lymphadenitis (KNAPP, 1963, 1968; WETZLER and HUBBERT, 1968).

Little is known about the fate of injected *Y. pseudotuberculosis* in experimental animals but it seems probable that cells of this species, like those of *Y. pestis*, are rapidly removed from circulation via the reticulo-endothelial system. RICHARDSON and HARKNESS (1970) showed that the organisms grow rapidly in dispersed rabbit spleen cells in 5 % $CO_2$ and 95 % $O_2$; slower growth is obtained in monolayered cells. The bacteria grow within compartments in kidney cells which sometimes nearly fill the cytoplasm whereas growth within spleen cells is more limited. No distinctions have been noted between the abilities of *vwa*$^+$ and *vwa*$^-$ organisms to parasitize cells of the rabbit. It was noted, however, that the response of the rabbit to infection resembles that of the guinea pig where the ability to produce V and W is not essential. The interesting possibility was thus raised that the virulence antigens are essential for the expression of acute but not chronic disease.

## C. Immunity

Protection provided by antibodies to fraction 1 might be opsonic in nature or could possibly be mediated via the action of complement. The same might be said for antibodies to V antigen; however, in this case the possibility also

exists that specific antibodies might inhibit some enzymatic process that is associated with Ca-dependence and related phenomena that seem to be required for the expression of virulence. These possibilities are amenable to experimental testing and further work in this area may supply new information regarding the role of the *vwa*$^+$ determinant.

At least one other immunogenic determinant exists that cannot be equated with V or fraction 1 antigen. This substance, which is shared by *Y. pestis* and *vwa*$^-$, *fra*$^-$ *Y. pseudotuberculosis*, provides long-term protection against plague (THAL, 1955). Chemical characterization of this immunogen has been hampered by its association with insoluble cell debris although a particulate preparation has been prepared by sonication and treatment with mild alkali (KEPPIE et al., 1958). Further purification has been complicated by the presence of lipopolysaccharide which yields short-term protection (LAWTON and SURGALLA, 1963). The nature of this third immunogen, which might conceivably promote cellular immunity, has not yet been determined.

## V. Meiotrophy

Many of the properties which distinguish *Y. pestis* from *Y. pseudotuberculosis* have been discussed in previous sections. These differences, plus some additional distinctions, are summarized in Table 2. Numerous reports have appeared which claim that *Y. pestis* can mutate to a form similar or identical to *Y. pseudotuberculosis* upon treatment with bacteriophages, exposure to antibiotics, or storage in the cold (*see* BRUBAKER et al., 1965b). Conversion of phenotype was also reported to occur under the influence of antisera directed against enteric bacteria (MIKHAILOVA and BEKKER, 1966). Certain pleiotrophic mutations are known to occur in bacteria which result in dramatic changes; events of this type could conceivably account for the assumed conversion of *Y. pestis* to *Y. pseudotuberculosis*. However, attempts by this reviewer to obtain single-step mutants of genetically marked *Y. pestis* which resemble *Y. pseudotuberculosis* have never been successful. It is possible, however, to obtain single-step mutants of *Y. pestis* which acquire individual characteristics of *Y. pseudotuberculosis*.

The first case of this type was the isolation by ENGLESBERG (1957a) of mutants of *Y. pestis* that ferment rhamnose. Such mutants were termed meiotrophs and, in the case of ability to utilize rhamnose, arose at the low rate of $2.6 \times 10^{-11}$. Subsequent study disclosed that wild-type *Y. pestis* yields similar melibiose meiotrophs (*unpublished observations*) as well as mutants that acquired the ability to assimilate low levels of $NH_4^+$, synthesize glycine, and hydrolyze urea (BRUBAKER and SULEN, 1971). Phenylalanine- and methionine-meiotrophs were described by ENGLESBERG and INGRAHAM (1957) and BROWNLOW and WESSMAN (1960). Further investigation may disclose that other functions can be restored in *Y. pestis* by meiotrophic mutation.

The nature of the mutational events that result in the acquisition of new properties has not been defined but the results of ENGLESBERG (1957b) and DODIN and BRYGOO (1959) indicate that regulatory functions in rhamnose-meiotrophs are normal. Attempts to induce meiotrophy by use of alkylating agents have been unsuccessful (*unpublished observations*); however, the possibility remains that meiotrophy might occur as a result of frameshift mutations. In any event, it seems reasonable to assume that *Y. pestis* has lost various ancillary functions during its recent evolution as an obligate parasite and that these functions were retained in *Y. pseudotuberculosis*. The ability to utilize rhamnose or synthesize methionine evidently confers no selective advantage to the former in its closed flea to-rodent cycle whereas the corresponding genes may facilitate the survival of *Y. pseudotuberculosis* in soil and water. The interesting point is that defective genes in *Y. pestis* are faithfully replicated indicating that the organisms lack an efficient mechanism to eliminate superfluous DNA. As an exercise, it should be possible to select for individual gain and loss mutations in *Y. pestis* and thereby acquire an organism which would closely resemble *Y. pseudotuberculosis*. Such conversion would not be expected to occur in a single-step.

## VI. Yersinia enterocolitica

GILBERT (1933) first characterized *Y. enterocolitica*, also termed by others as *Bacterium enterocoliticum* and *Pasteurella* "X", from isolates of human origin. Certain strains of this species are of high virulence in pigs, chinchillas, and hares but not in conventional laboratory animals (MOLLARET and GUILLON, 1965). The pathogenicity of *Y. enterolitica* for man remains uncertain although the organisms have been isolated routinely from patients with acute abdominal disease (NILÉHN and SJÖSTRÖM, 1967) and have been associated with arthritis (*see* NILÉHN, 1969; AHVONEN et al., 1969b), other forms of chronic disease, and acute infections (*see* NILÉHN, 1969; RUSU, 1970). The proposed role of yersiniae in causing acute regional ileitis has been seriously questioned (KNAPP et al., 1970).

*Y. enterocolitica* exhibits a marked but perhaps superficial resemblance to *Y. pseudotuberculosis* with respect to cultural and diagnostic determinants. The species are distinguished by the ability of *Y. enterocolitica* to ferment cellobiose, and, depending upon the biotype, to ferment sucrose, sorbose, and to decarboxylate ornithine. In contrast, *Y. enterocolitica*, unlike *Y. pseudotuberculosis*, generally fails to ferment salicin or hydrolyze aesculin (NILÉHN, 1969). BURROWS and GILLETT (1966) showed that cells of *Y. enterocolitica* often exhibit a nutritional requirement for thiamine at 28° C and all strains examined by these workers require thiamine and either cystine or methionine for growth at 37° C. Little is known about the intermediary metabolism of this species.

Strains of *Y. enterocolitica* have been distinguished on the basis of 9 serotypes (WINBLAD, 1968) which correspond to some degree with host origin and

the 5 biotypes and 5 temporate phage types of NILÉHN (1969). Lysogeny is common in *Y. enterocolitica* as opposed to other members of the genus, although production of defective phage particles by *Y. pseudotuberculosis* has been reported (ARKHANGEL'SKAIA and VOROB'EV, 1968). There is little or no antigenic relationship between *Y. enterocolitica* and the other yersiniae although certain strains of the former cross-react with brucellae (AHVONEN et al., 1969a). On the other hand, a few strains of *Y. enterocolitica* are sensitive to pesticin I (*unpublished observations*). It is evident from these observations that *Y. pestis* and *Y. pseudotuberculosis* are more closely related to each other than they are to *Y. enterocolitica* which might be more properly placed in another genus. Perhaps studies of base homologies will permit a more precise assessment of the taxonomic position of *Y. enterocolitica*.

## VII. Comments

> "... the plague bacillus never dies or disappears for good, it can lie dormant for years and years in furniture and linen-chests and it bides its time in bedrooms, cellars, trunks, and book-shelves ..."
>
> Albert Camus in *The Plague*

Although plague is not presently a major epidemic disease, *Y. pestis* is now more firmly entrenched in endemic foci throughout the world than at any time in its past (BALTAZARD, 1960). The intervention of man in such foci frequently leads to isolated cases, as in western North America, or to severe epidemics such as those in Vietnam (TRONG et al., 1967). With the passage of time the latter may occur with increasing number and severity throughout the world as the human population becomes larger and more mobile. The fact that plague has had irregular periods of quiescence and recrudescence in the past has prompted many epidemiologists to view the future with some pessimism although it is most unlikely that a pandemic could again occur in the absence of other major social catastrophies. However, any severe and prolonged breakdown in sanitation and public health would result in the eventual appearance of plague unless the afflicted area was well isolated from endemic foci. Accordingly, it seems prudent to continue the development of improved methods of immunization, rodent control, and antibiotic therapy.

In pursuit of these goals, many investigators have made important contributions relating to basic processes of both the host and parasite. Discoveries of this nature are to be expected in any investigation where inhibitors, even subtile inhibitors such as pathogenic bacteria, are used to isolate a biochemical reaction from the steady state. The consequences of infecting the sensitive host are dramatic, indicating interference with reactions that are not fully understood but which are obviously essential to life. As noted in this review, various metabolites produced by yersiniae directly or indirectly block diverse reactions such as growth of tumor cells, reduction of coenzyme Q, and initiation of

septation and DNA synthesis. Further study of these and other phenomena caused by bacterial parasites should eventually lead to a better biochemical definition of virulence. Of more importance, such investigations will almost surely result in a better understanding of the biology of the uninfected host.

Table 1. *$LD^{50}$ of wild-type cells and various avirulent mutants of Yersinia pestis in mice and guinea pigs*

| Genotype[a] | | | | | Intraperitoneal $LD^{50}$ | |
|---|---|---|---|---|---|---|
| *vwa* | *fra* | *pst* | *pgm*[b] | *pur*[c] | mouse | guinea pig |
| + | + | + | + | + | $<10$ | $<10$ |
| 0 | + | + | + | + | $>10^7$ | $>10^8$ |
| + | 0 | + | + | + | $<10$ | $\sim10^4$ |
| + | + | 0 | + | + | $\sim10^5$ | $\sim10^6$ |
| + | + | + | 0 | + | $>10^7$ | $>10^8$ |
| + | + | + | + | 0 | $\geq 10^2$ | $\geq 10^4$ |

[a] *vwa*$^+$ = ability to produce V and W antigens; *fra*$^+$ = ability to produce envelope or fraction 1 antigen; *pst*$^+$ = ability to synthesize pesticin I, coagulase, and fibrinolysin; *pgm*$^+$ = ability to absorb certain pigments; *pur*$^+$ = ability to synthesize purines *de novo*.

[b] Certain strains of mice may be less resistant to *pgm*$^-$ cells.

[c] Late blocks in *de novo* synthesis result in a greater degree of avirulence than do early blocks.

Table 2. *Determinations which serve to distinguish between wild-type Yersinia pestis and Yersinia pseudotuberculosis*

| Determinant | *Y. pestis* | *Y. pseudotuberculosis* |
|---|---|---|
| Fermentation of rhamnose | 0[a] | + |
| Fermentation of melibiose | 0[a] | + |
| Expression of urease | 0[a] | + |
| Expression of glucose 6-phosphate dehydrogenase | 0 | + |
| Expression of adenine deaminase | 0 | + |
| Motility at 20° C | 0 | + |
| Synthesis of methionine | 0[a] | + |
| Synthesis of phenylalanine | 0[a] | + |
| Synthesis of glycine | 0[a] | + |
| Assimilation of low levels of $NH_4^+$ | 0[a] | + |
| Production of pesticin I, coagulase, and fibrinolysin | +[b] | 0 |
| Production of envelopes | +[b] | 0 |
| Expression of murine toxin | +[b] | 0 |

[a] Determinant reported to undergo meiotrophic gain mutation.

[b] Determinant reported to undergo mutational loss.

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# Host Genotype and Antibody Formation

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In a group of randomly selected individuals of a given species the same dose of antigen will provoke immune responses differing in quantity as well as in quality. This variation of the immune response has two possible reasons: it reflects differences in 1) the individual's pre- or postnatal history and experience with antigens, encountered in his surroundings and/or 2) the genetic constitution of the individual, that is the capacity to respond to a given antigen is genetically determined and the response is only modified by antigenic experiences. The role of the genetic constitution is especially apparent when one compars immune responses to a given antigen in a homogeneous and heterogeneous population. Genetically uniform individuals of an inbred strain respond in a very uniform way to a strong and repeated antigenic stimulus which provokes maximal immune responses. In non-inbred strains the same immunization leads to a wide variation in antibody titres. This uniformity in antibody response in inbred strains, apparent mainly after maximal antigenic stimulation, shows that variability resulting from personal history manifests itself predominantly in reactions to weak stimuli (where previous contact with cross-reacting antigens plays a decesive role).

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Immune response to antigenic stimulation is a complex phenotypic reaction which begins at the moment of antigen penetration into the organism and ends with the production of antibodies and/or of sensitized cells directed against the antigen. It is obvious that the individual steps of such a complicated process have to be regulated in some way. To trace the role of the genetic regulation one of the possible approaches is to look for defects in the immune reaction and to compare them with normal processes of antibody production and secretion. These abnormalities might be based on defects in different steps of the immune reaction and not all of these defects must necessarily be connected directly with the genetically determined immune constitution of the organism but may involve some of the non-specific steps, such as hormonal disbalances. For this reason it is important to analyze the individual steps of the immune reaction and to prove that the deficiency of the response is caused by an inadequacy of the immune apparatus itself.

The control of the immune response involves two possible ways, *active* or *passive* control. (CINADER et al., 1969.) *Active* control means that the synthesis of a certain antibody immunoglobulin is directly controlled by a gene or genes present in the cell which is making and secreting this immunoglobulin. The capacity to form different antibodies reflects the presence of different genes which are available to the producing cell. Changes or absence of some genes (structural and/or regulatory) are responsible for a change in the capacity to form the corresponding antibody. Another type of active control might operate in the antigen-processing step, if such a step is necessary for antibody formation.

*Passive* control is based on the fact that under normal conditions the organism does not react against his own antigenic components. In case of cross-reactivity between an antigen and antigenic constituents of the organism, the antibodies formed usually are only against foreign antigenic determinants. Since the antigenic composition of an organism is the result of differentiation and is determined by the genetic constitution of the organism, the genetic control of this antigenic composition simultaneously determines which antibody should not be produced.

The inability of an organism to react against an antigen may therefore either be a phenotypic expression of the lack of a gene controlling this particular antibody structure or it may be caused by the presence of a similar antigenic structure that is part of the responding organism.

## A. Genetic Control of Resistance to Infection

It is a well known phenomenon that certain species are resistant to some infection while the same infection is higly pathogenic in other species. Thus rats are higly resistant to diphteria infection compared to man or guinea-pig who are very susceptible to this infection. This type of resistance is a characteristic feature of a species. Another example is the susceptibility to Salmonella infections. *S. typhi* is pathogenic in man, *S. gallinarum* causes infection only in chicken and *S. typhi-murium* is highly pathogenic for mice but does not infect rats or other species (ROWLEY, 1969).

It has been proved repeatedly (GUYER and SMITH, 1923; SCHEIBEL, 1943; GOWEN, 1948) Fig. 1, that there is a direct connection between resistance or susceptibility to an infection and the genetic constitution of the organism. It seems that in resistance to infection passive control of the immune response plays an important role (ROWLEY and JENKIN, 1962). There is a high structural similarity and cross-reactivity between the surface antigens of bacteria and surface structures on the cells of the host (SIMONSEN and HARRIS, 1956; MARKOWITZ et al., 1960). For example, the blood substance antigens can also be found on bacteria, so that in individuals of a particular blood group the response to bacteria with the same antigen is lower or lacking completely.

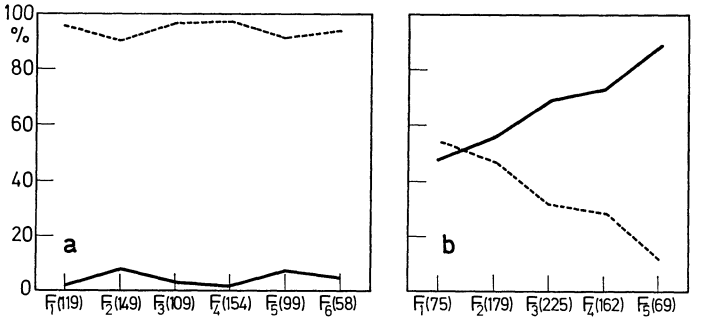


Fig. 1. Curve a represents results of crosses among good antibody producers and curve b results of crosses among poor antibody producers. The vertical axis indicates the percentage of offspring classified according to the amount of antibody produced. The generation number is plotted along the horizontal axis with the number of offspring in parenthesis. The cut-off for classifying an individual as a producer -------- or non-producer ——— is at 0.001 antitoxin units per ml. (From SCHEIBEL, 1943)

Resistance to infection is not always connected with immune mechanism. For example, patients with sickle cell anemia are highly resistant to malaria. The erythrocytes in sickle cell anemia contain abnormal haemoglobin, Hb-S, which is responsible for the disease. The disposition to form this haemoglobin is heritable and is transferred from parents to children. Resistance to malaria in these patients is not a result of a more efficient immune response but is caused by the abnormal Hb-S haemoglobin which is not metabolized by the malaria parasite.

Other forms of resistances are dependent on neutrofil leucocytes and on phagocytic and degradative activities of macrophages without any necessary intervention of antibodies or specifically sensitized cells. Resistance or sensitivity to infection depends also on anatomic differences or on differences in the biochemical processes which again are genetically determined. In chickens, two strains have been described differing in susceptibility to *S. pullorum* infection. The only demonstrable differences was that in the resistant strain there was a much higher number of lymphocytes in blood and tissues than in the susceptible strain. After splenectomy, which lowered the number of lymphocytes circulating in the blood of the resistant strain the susceptibility

of this strain increased and became similar to that of the susceptible strain. In the latter splenectomy was without effect.

In resistance to infection humoral factors play a considerable role and this applies especially to antibodies. Low levels of actively formed antibodies result in higher susceptibility to infection. In the first step of defence against many types of infections the fate of the infectious agent after penetration into the organism depends on the level of appropriate pre-existing antibodies in the blood and tissue fluids (Rowley, 1969). Here so called "natural" antibodies play a prominent role; these antibodies reflect the capacity of different species to react to different antigens in their environment. High susceptibility to infection in some strains of mice is caused by a decreased ability to form the corresponding antibodies; this is a genetic trait and as such is transferred from parents to their offspring (Carlianfanti, 1948).

## B. The Genetic Control of the Antibody Response

Antigen which comes into contact with the cells of the lymphatic system induces an immune reaction. This is characterized by the appearance of cells which synthetize and secrete antibodies and of cells which take part in the cellular immune reaction. These reactions involve a participation of different types of cells. All of these cells acquire their capacities for participation in immune reactions by differentiation. Differentiation is controlled by numerous genes, some of them acting in the cells destined to produce and secrete antibodies, other genes control the activity of cells that participate in non-specific but nevertheless important steps in the immune reaction. It is relatively simple to ascertain if the genetic mechanism operates in cells of the lymphatic organs. Transfer of lymphatic tissue to irradiated, non-responding recipients can help to decide if the responsible reaction exists in the transferred cells. This approach was used in the study of many genetic differences in antibody responses and a direct connection between transferred cells of the lymphatic system and the character of the transmitted response was shown (McDevitt and Tyan, 1968; Foester et al., 1969; Říha and Škárová, 1969).

It is much more difficult to decide whether the genetic control operates directly in the cells that actively synthetize antibodies or in other cells of the lymphatic system. The isolation of pure physiologically active cell lines is so far very difficult and thus information based on the transfer of pure cell lines is lacking. In two inbred strains of guinea-pigs it was shown that macrophages are not the cells responsible for differences in the immune response to poly-L-lysine (Paul et al., 1969). In inbred strains of mice the antibody response to the haptenic p-aminobenzoic acid group can be transferred to a non-responding strain by bone marrow cells from a well responding strain whereas thymocytes play no decisive role (Říha and Škárová, 1969). The capacity of the immune response to synthetic polypeptide antigen (T, G)-A--L can be transferred to poorly responding $C_3H$ recipients with spleen cell suspensions from highly responding ($C_3H \times C57Bl/6$) $F_1$ hybrid mice (McDevitt and Tyan, 1968).

The cells responsible for this genetic control are also present in fetal liver (TYAN et al., 1969). These experiments prove beyond doubt that some part of the genetic control of the immune response resides in cells of the lymphatic tissue where it most probably operates in the synthesis of the antibody molecule.

### 1. Genetic Regulation of the Synthesis of the Constant Part of the Immunoglobulin Polypetide Chains

The first immunoglobulin thoroughly studied, the IgG with a molecular weight of 160000, was found to consist of molecules formed by two types of polypeptide chains. Each molecule contains two identical light chains (L)

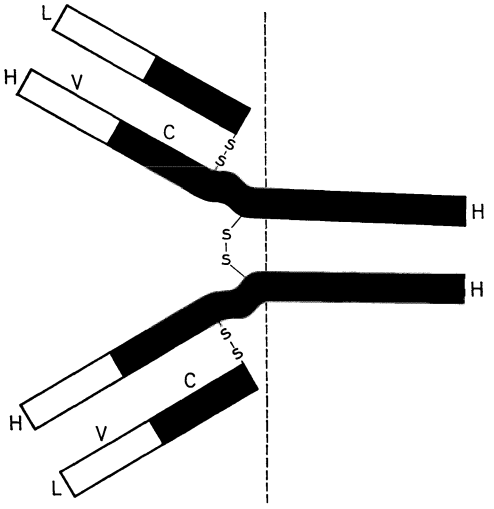


Fig. 2. Schematic model of an immunoglobulin molecule

m. w. 22000 and two identical heavy chains (H) m. w. 55000 which are bound together by covalent disulphide bridges and by non-covalent hydrophobic bonds (EDELMAN and POULIK, 1961; COHEN and PORTER, 1964; EDELMAN and GALLY, 1964). This four-chain structure was found later to be typical for all classes of immunoglobulins and even the high molecular weight immunoglobulins (IgM and the secretory form of IgA) could be dissociated into typical four-chain subunits (Fig. 2). Every immunoglobulin-producing cell must therefore form at least two types of polypeptide chains and, accordingly, contain at least two structural genes controlling the synthesis of these chains. When the first amino acid sequences of immunoglobulin chains were determined it was found that both chains, L and H, have two regions, one which is highly variable, the V region, varying from chain to chain, and a constant region, the C region, which is typical for chains of a certain type or class of immunoglobulins.

The constant region of the H chain occupies the C terminal end of the chain and is the same in all heavy chains of a given immunoglobulin class,

subclass and allotype. The variable region, which occupies one quarter of the H chain lengths from the $NH_2$ end, is responsible for differences among individual molecules of an immunoglobulin class or subclass. Because the combining site of an antibody is located on the N-terminal end of the chains, it seems most likely that the variability of the N-terminal end of the heavy chain is a reflection of the different antibody specificities of immunoglobulin

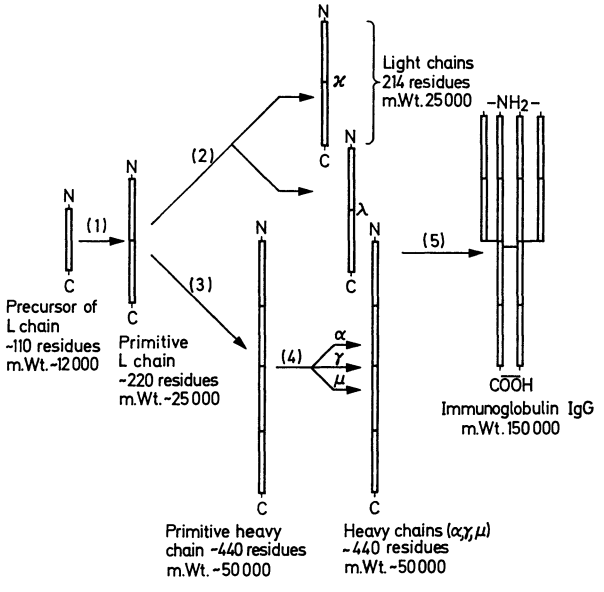


Fig. 3. A tentative scheme for the genetic origin of immunoglobulins. (From HILL et al., 1966)

molecules (PORTER, 1967). The L chain also consists of a variable and a constant region (HILSCHMAN and CRAIG, 1965; PUTNAM et al., 1967). The constant region forms the C-terminal half and the variable (V) region the N-terminal half of the L polypeptide chain. As in the H chain, the constant region of the L chain is common for all immunoglobulin molecules of the same type while differences among L chains of the same type are caused by different amino acid sequences of the variable L chain region. The variable regions of H and L chains have a similar length while the constant region of the H chain is three times as long as in the L chain. Because comparison of amino acid sequences and positions of disulphide interchain bridges in human and mouse L chains, in human H chains and the $F_c$ portion of rabbit H chain shows a striking similarity (SINGER and DOOLITTLE, 1966; PUTNAM et al., 1967; HILL et al., 1967; EDELMAN, 1967), it is postulated that the respective genes are descendants of a primitive gene which controlled the synthesis of a polypeptide chain corresponding to the constant region of the present L chain. By duplication of this primitive gene during phylogeny a new genetic region was formed which is responsible for L chain synthesis and, subsequently by another duplication, the gene for the H chain arose. During this development mutational changes led to the present diversity of the immunoglobulin chains (Fig. 3).

The constant regions of both polypeptide chains carry so called allotypic specificities. Allotypes were described in man and rabbits nearly simultaneously and later also in other mammalian species. Especially well known are the allotypes in mice. Allotypes are genetically determined markers on immunoglobulin molecules and are responsible for individual differences. They represent a special amino acid combination forming a determinant group on the immunoglobulin molecule surface. The determinant group may be formed by variation in one amino acid only or by changes in a number of different amino acids (BAGLIONI et al., 1966; THORPE and DEUTSCH, 1966; FRANGIONE et al., 1966). The rest of the constant region of the polypeptide chains is the same in all individuals of a species and represents the species-specific region of the immunoglobulin molecule.

The genes responsible for the existence of allotypic determinants were deduced on the basis of family studies and in typical crossing experiments these genes are in fact structural genes that control the synthesis of the constant region of H and L chains as the allotypic determinants are located in this region of the chains. These genes form a multiple allelic series, that is they exist in more than two alternative forms or allels. The existence of such multiple allelic genes is one of the typical features of immunoglobulin synthesis control since usually, but certainly not always, genes exist only in two allelic forms.

Allotypic specificities are present both on H and L chains. These located on the L chain are present on molecules of all immunoglobulin classes containing the respective L chain, while the H chain allotypes are typical for a certain class or subclass of immunoglobulins.

In most vertebrates the L chains exist in two forms which differ in their amino acid sequence in the constant region. These two types of L chains are called kappa and lambda and in man are represented among all immunoglobulin molecules of each individual, usually constituting 60% and 40% respectively (FAHEY, 1962; COHN and MILSTEIN, 1967). In man the so called InV allotypes of the kappa light chain have been described and it seems that other allotypic differences also exist in lambda chains. According to the InV allotypic specificities, the genes controlling the synthesis of kappa chains in humans are called InV (1, 2 or 3).

In man the allotypic factors located on the constant part of the IgG heavy chain (gamma chain) are described as Gm factors and the genes controlling their synthesis accordingly are known as Gm genes. IgG molecules exist in four forms characterized by different antigenic determinants. They are present in every individual and are therefore called subclasses and described as $IgG_1$ (until recently called We or $gamma_{2b}$), $IgG_2$ (Ne or $gamma_{2a}$), $IgG_3$ (Vi or $gamma_{2c}$) and $IgG_4$ (Ge or $gamma_{2d}$). Each of these subclasses has its own allotypic determinants (KUNKEL et al., 1964; 1966) which are located in different positions on the constant part of the H chains. For $IgG_1$ the Gm factors Gm [1(a), 2(x), 4(f), 8(e), 9(p), 17, 18, and 22]; for $IgG_2$ the Gm [8(e), or 23]; for $IgG_3$ Gm [5(b, $b_1$), 6(c), 10($b^{alfa}$), 11($b^{beta}$), 14($b_4$), 15, 16, and 21] are characteristic. No allotypic variants have yet been described in the $IgG_4$

subclass. Allotypic specifities are located in different regions of H chains and therefore the occurrence of two or more allotypic determinants on one chain is possible. For example, Gm(a) and Gm(y) are present on the $F_c$ fragment (HARBOE et al., 1962) while Gm(f) and Gm(z) are present on the $F_d$ fragment (POLMAR and STEINBERG, 1964). This means that the subclass $IgG_1$ is controlled by a series of allelic genes which for the Gm factors (a and y) and (z and f) is represented by genes Gm(az) and Gm(fy). The product of each of these genes, the immunoglobulin $IgG_1$ H chain, has two genetic markers, located on different parts of the H chain (Fig. 4) (LITWIN and KUNKEL, 1967). The same applies to Gm(b) allotypic markers present on the $IgG_3$ subclass.

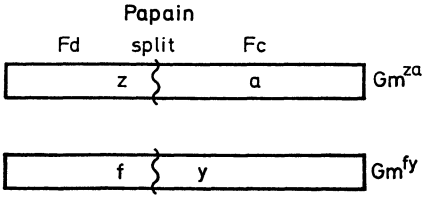


Fig. 4. Diagrammatic representation of the localization of Gm antigens on γGl-heavy chains. The two major types of heavy chains found in Caucasians are shown, with the positions on the $F_d$ and $F_c$ fragments of each pair of genetic antigens. The allelic genes controlling these chains are identical on the right (From LITWIN and KUNKEL, 1967).

| | Caucasoid | | | | Negroid | | | | Mongoloid | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | x | | | | | | | | | x | a |
| γG1 | a | a | y | y | a | a | a | a | a | a | a | y |
| | z | z | f | f | z | z | z | z | z | z | z | f |
| γG3 | g | g | $b^0$ | $b^0$ | $b^0$ | $b^0$ | $b^0$ | $b^0$ | $b^0$ | g | g | $b^0$ |
| | | | $b^1$ | $b^1$ | $b^1$ | $b^1$ | $b^1$ | s | st | | | $b^1$ |
| | | | $b^4$ | $b^4$ | $b^4$ | | $b^4$ | | | | | $b^4$ |
| | | | $b^5$ | $b^5$ | $b^5$ | $c^5$ | $b^5$ | $b^5$ | $b^5$ | | | $b^5$ |
| | | | $b^3$ | $b^3$ | $b^3$ | $c^3$ | $b^3$ | $b^3$ | $b^3$ | | | $b^3$ |
| γG2 | | | n | | | | | | | | | n |

Fig. 5. Gene complexes in different racial groups arranged in vertical fashion according to the subgroup of heavy chains involved. (From LITWIN and KUNKEL, 1967)

Allotypic factors present on the subclasses of IgG are transmitted as complexes which are typical for ethnic groups (Fig. 5). The existence of these complexes shows that crossing-over among genes for IgG subclasses is very rare and that genes for the four subclasses of human IgG are closely linked, probably in the sequence $IgG_4$—$IgG_2$—$IgG_3$—$IgG_1$ (KUNKEL et al., 1964, 1966). Similarly, the genes for the constant region of the light chain are also closely linked (MÅRTENSSON, 1966; LENNOX and COHN, 1967). But no linkage was found between the genes for H chains and for L chains and it seems that they are even located on two different chromosomes (FUDENBERG et al., 1963).

In rabbits, so far, 14 allotypic specificities have been described. On the L chains allotypes A4, A5, A6, and A9 are present; others (A1, A2, A3) are located on the $F_d$ fragment of the H chain (STEMKE, 1964). The specificities A1, A2, A3 are determined by allelic forms of the gene *a* and allotypes A4, A5, A6, and A9 are controlled by alleles of the gene *b* (DUBISKI et al., 1959; OUDIN, 1960a, b; DRAY et al., 1962; DUBISKI and MULLER, 1967). Other specificities, A7 and A8 (NISONOFF and THORBECKE, 1964; HAMMERS et al., 1966) and specificity A11 (MANDY and TODD, 1968) are also present on the H chain but are controlled by different genes.

In mice, like in man and rabbits, allotypic differences in immunoglobulins have been described. The study of these markers was greatly facilitated by the existence of inbred strains consisting of homozygous, genetically identical individuals. Each inbred strains is, therefore, characterized by a combination of allotypic markers. Attempts to located the genes for immunoglobulins by reference to other known genes has so far had only limited success. No linkage was found between immunoglobulin genes and other genes with known chromosome locations (chromosomes: Ic, p; IId, se; IIIs, hr; IV Se; V A, Ra, Sd; VI Ca; VII Re; VIII b; IX H-2, Ir-1; XI $Mi^{wh}$; XII ep, ru; XIII Lp; XIV Sa; XV ax; XVI Va; XVIII Os, Ea-1; XX sex (HERZENBERG et al., 1968).

Immunoglobulin molecules also contain a carbohydrate moiety which plays a role in the transport of the finished immunoglobulin molecules across the cell membrane. For the formation and attachment of this carbohydrate group some other genes are necessary which also participate in the normal function of the immunoglobulin-forming apparatus.

A restriction of the potentiality of the cells is typical for immunoglobulin-synthetizing cells. Although the cell may contain genetic information for the production of all light and heavy chains of different types of classes and subclasses of immunoglobulins in two allotypic forms, the final product of a given cell's activity is one homogene ousprotein e. g. only one gene for the H chain and one gene for the L chain is active. This type of regulation ("allelic exclusion") is unique for immunoglobulin synthesis (PERNIS et al., 1965; CEBRA et al., 1966).

## 2. The Genetic Control of Antibody Specificity

Comparing immune responses to different antigens in a group of inbred animals it was found that the response varies greatly and that such variations are controlled by genetic factors. FINK and QUINN (1953) have shown that the antibody response to sheep erythrocytes, BSA, ovalbumin or pneumoccocal polysaccharide is dependent on the genetic constitution and that among inbred strains of mice the level of antibody formed differs and is characteristic for each strain. The same conclusion was drown by DAVIDSOHN and STERN (1949) from experiments in mice immunized with sheep erythrocytes.

A number of model systems have been reported for genetically determined qualitative or quantitative differences in immune responses in different animal species. Analyzing the genetic basis of control, it was shown that such differen-

ces involve either polygenic control as in reaction to synthetic antigens (McDevitt and Sela, 1965; Simonian et al., 1968), to viruses (Sang and Sobey, 1954; Lennox, 1966) and to different types of erythrocytes (Playfair, 1968a), or the differences were controlled by a single gene as in model systems in which simple, usually synthetic antigens, were used (Benacerraf et al., 1963; Pinchuck and Maurer, 1965; Pinchuck and Maurer, 1968).

The polygenic basis of control of the immune response to complex antigens may represent only the sum of monogenic control of antibodies to individual determinants on the complex molecules. Since all antibodies are formed at the same time, all the individual genes must be assumed to be activated simultaneously. Another reason for polygenic control might be the involment of several cell types in the immune reaction, for example cells responsible for the processing of the antigen.

A typical antigen is formed by a molecule carrying on its surface a set of different antigenic determinants. Therefore, the antibodies formed to such an antigen are heterogeneous since they represent a mixture of antibodies with different specificities directed against individual determinants. The antigenic capacity to form antibodies of a certain specificity can hardly be tested with such a complex antigen since the lack of response to one determinant may be masked by the presence of antibodies to the other determinants. For this reason, to measure the capacity to form antibody of a known specificity, well defined determinant groups, usually simple haptens attached to a protein carrier molecule or to a synthetic antigen must be used.

## a) Genetic Regulation of Antibody Responses to Simple Antigenic Determinants

In recent years a number of data have accumulated that elucidate the genetic basis for differences in the immune response in a number of animal species (Arquilla and Finn, 1965; Gill, 1965; Pinchuck and Maurer, 1965; McDevitt and Sela, 1965; Škárová et al., 1966; Ben-Efraim and Leskowitz, 1966; Ben-Efraim et al., 1967; Simonian et al., 1968; Warner et al., 1968; Škárová and Říha, 1969; Říha and Škárová, 1969).

Arquilla and Finn (1963, 1965) were the first to show that two higly inbred strains of guinea-pigs differ in their immune response to insulin. Both strains produce anti-insulin antibodies, but strain 13 forms antibodies reacting with the C-terminal end of the insuline polypeptide chains, whereas strain 2 produces antibodies specific for the N-terminal end of insulin A and B chains.

Linear polymers consisting of one L-alpha-amino acid only (lysine or glutamic acid) or random copolymers of two such amino acids (glutamyl-lysine, glutamyl-alanine or lysyl-alanine) are not immunogenic for mice but copolymers of as few as three amino acids are antigenic in most inbred strains of mice. A copolymer, formed predominantly by glutamyl-lysine with only 5 % of alanine, induces antibody formation only in inbred strains $C_3H$ and Balb/c but not in strains C57Bl, A/J or CBA (Pinchuck and Maurer, 1965).

The latter strains do not form detectable antibodies against this copolymer at all or only in minute quantities. It was found that this response is genetically controlled. The offspring of non-responding parents are also non responders. On the other hand, when at least one parent belongs to the responding strain, the offspring in the $F_1$ will also be responders. According to the authors, this response is controlled by a single autosomal dominant Mendelian factor. It is interesting that such differences were noted only when a copolymer with 5% alanine was used, whereas a copolymer containing 10% alanine residues was antigenic in all strains tested. The effect of this increase in the alanine content of the copolymer can best be explained by the formation of new types of determinants; the complexity of the random copolymers allows for different combination of the three amino acid residues which, in turn, results in the formation

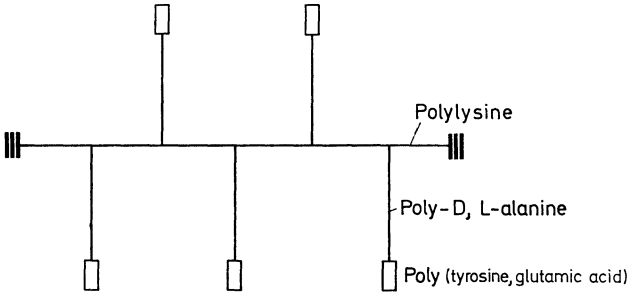


Fig. 6. Schematic diagram of the structural pattern of (T,G)-A–L 509. (From McDevitt and Sela, 1965)

of determinants of different structure. The presence of different determinants on the copolymer surface provides more opportunities for the formation of antibody to at least some of the determinants.

McDevitt and Sela (1965) have shown that in inbred strains of mice the capacity to repsond to a synthetic, multichain polypeptide antigen carrying only a restricted number of antigenic determinants is under genetic control. The antigens that have been used are composed of a poly-L-lysine backbone with side chains of poly-D,L-alanine (Fig. 6). This molecule by itself is not immunogenic but becomes so when tyrosine and glutamic acid are attached to the poly-D,L-alanine side chain [(T, G)-A--L). The antibodies to this antigen are specific against the end amino acids tyrosine and glutamic acid. The determinants can also be changed by attaching, instead of tyrosine, phenylalanine, [(P,G)-A--L] or histidine [(H,G)-A--L]. In response to immunization with (T,G)-A--L, the C57 strain of mice forms about ten times more antibody than strain CBA. Responses to this antigen in the $F_1$ generation and following back crossing could best be explained by the existence of one major autosomal dominant factor with a possible modifying effect of some other factors (Fig. 7). The authors have designated this autosomal factor as Ir-1. When histidine is exchanged for tyrosine in the side chain of the basic poly-L-lysine chain, the immune response in these two inbred strains of mice is just the opposite from

that observed with after (T,G)-A--L. Strain C57 responds only poorly whereas strain CBA gives a good antibody response (McDEVITT and SELA, 1965, 1967). The third modification of the antigen (P,G)-A--L evokes a good response in both strains. $F_1$ hybrids of C57 and CBA strains respond well to all three antigens which means that the capacity for a good response is dominant to a

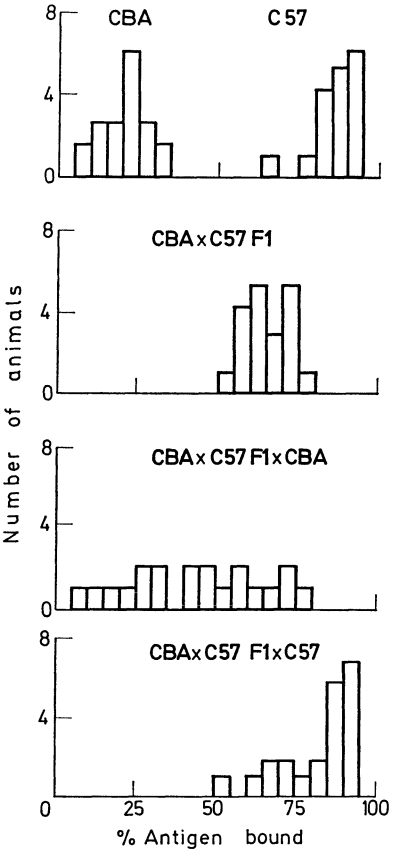


Fig. 7. Immune responses of mice given 10 μg (T,G)-A–L 509 in complete Freund's adjuvant, and boosted with 10 μg of the same antigen in saline. (From McDEVITT and SELA, 1965)

poor response. Because in all three cases the antigenic carrier is the same, and the only difference is in the structure of antigenic determinants, the likeliest explanation is that the genetic control responsible for the response to this antigen is specific for this antigen's determinants. Similar differences in antibody responses to these synthetic polypeptide antigens were described also in inbred strains of guinea pigs (BEN-EFRAIM et al., 1967).

Genetic control of the antibody response was observed also in DBA/1 and SJL strains of mice. When these two strains are immunized with a similar multichain polypeptide antigen (T,G)-Pro–L, mice of strain SJL give an excelent antibody response whereas DBA/1 mice respond poorly (MOZES et al.,

1969). The gene controlling the response to this antigen has been designated as Ir-3.

No significant qualitative differences between the antibodies formed by well responding and poorly responding strains were found and it, therefore, seems that the differences among the strains are only quantitative (McDevitt, 1968). There is no correlation between the type of response and participation of different immunoglobulin classes and allotypes in the antibody response. This proves that the latter responses are not dependent on the function of structural genes that control the C-terminal part of the immunoglobulin chain.

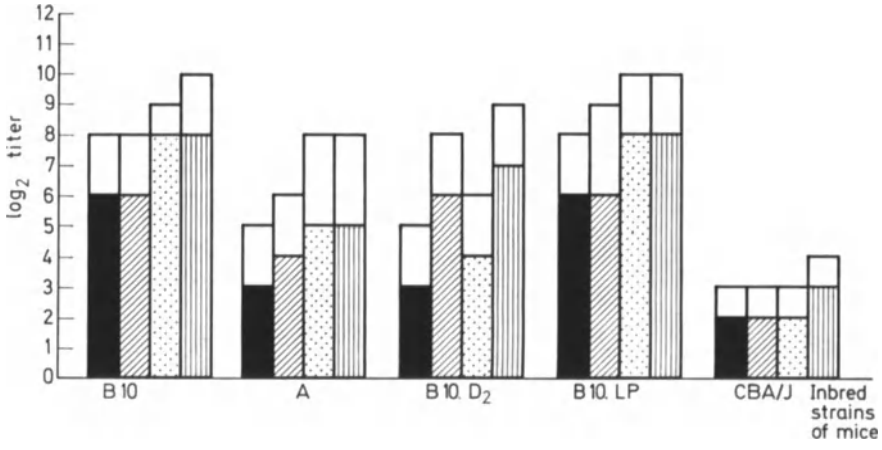


Fig. 8. Immune responses of inbred strains of mice A; C57Bl/10.ScSN; C57Bl/10.LP; C57Bl/10.$D_2$ and CBA/J against p-aminobenzoic and sulfanilic acid conjugated to BGG. ■ titer against p-aminobenzoic acid after immunization with p-aminobenzoic acid-BGG-sulfanilic acid. ▨ titer against sulfanilic acid after immunization with p-aminobenzoic acid-BGG-sulfanilic acid. ▩ titer against p-aminobenzoic acid after immunization with p-aminobenzoic acid-BGG. ▥ titer against sulfanilic acid after immunization with sulfanilic acid-BGG. ▭ titer in Coomb's test

Genetic control of the antibody response also was studied by analyzing responses to different haptenic groups (Škárová et al., 1966; Škárová and Říha, 1969; Říha and Škárová, 1969). We have shown that non-inbred rabbits immunized with p-aminobenzoic acid and sulfanilic acid bound to highly immunogenic bovine gamma globulin, differ in their response to these haptens; some are good or poor producers of antibodies to both haptens, but some of the experimental animals responded to one hapten only. In crossing experiments the ability to respond was transmitted as a dominant factor. For further study of the nature of this genetic control, we turned to inbred strains of mice (Škárová and Říha, 1969). We immunized five inbred and non-inbred strains of mice with different protein carriers to which two haptenic groups, p-aminobenzoic acid and sulfanilic acid had been conjugated. After immunization, two of the strains of inbred mice, A and CBA/J, responded only with low titres to p-aminobenzoic acid and sulfanilic acid, while in strains ScSN and

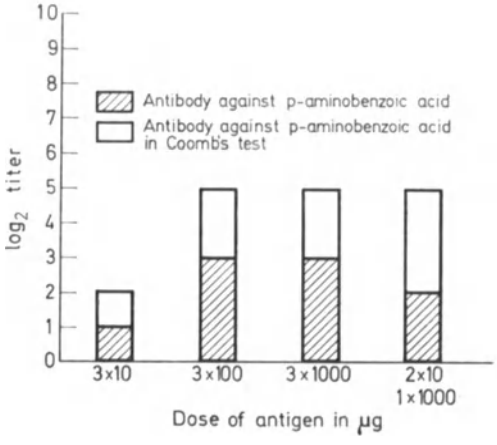


Fig. 9

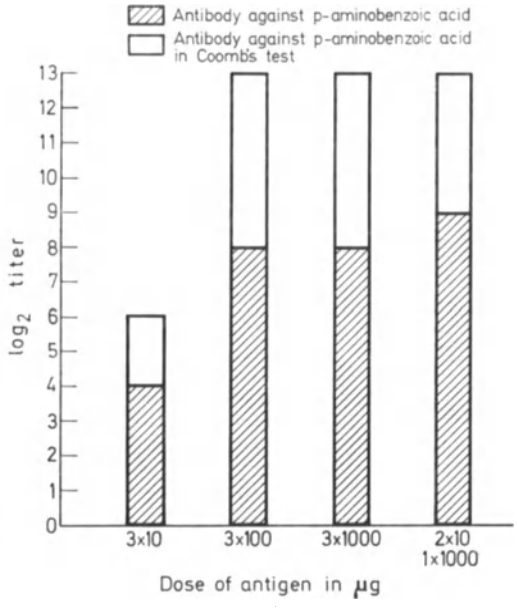


Fig. 10

Fig. 9 Influence of the dose of antigen on antibody responses in CBA/J mice

Fig. 10. Influence of the dose of antigen on antibody responses in C57Bl/10.LP mice

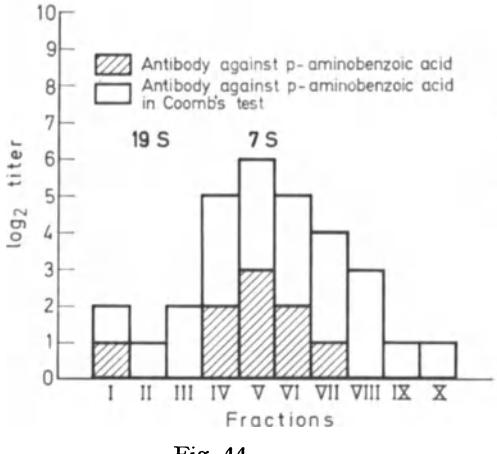


Fig. 11

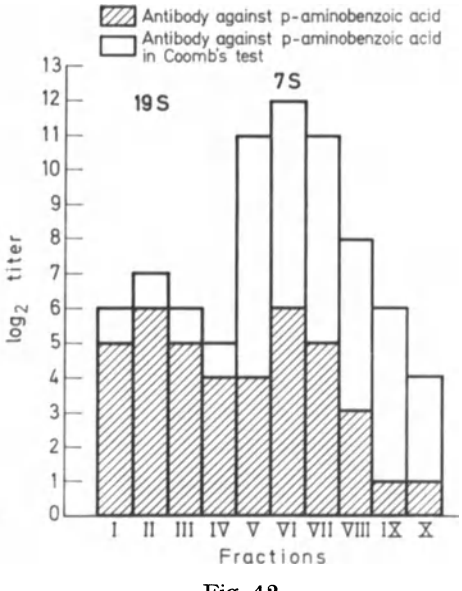


Fig. 12

Fig. 11. Distribution of 19S and 7S antibodies in fractions from sera of CBA/J mice after centrifugation in sucrose gradient

Fig. 12. Distribution of 19S and 7S antibodies in fractions from sera of C57Bl/10.LP mice after centrifugation in sucrose gradient

B10. LP, both anti-hapten antibodies reached direct haemagglutination titres of at least ten-fold magnitude. After immunization of strain B10.$D_2$, serum antibodies were mainly directed against sulfanilic acid, anti p-aminobenzoic acid antibodies being barely detectable. Indirect haemagglutination gave higher titres in all sera tested, but the differences among individual strains remained unchanged (Fig. 8).

These differences in anti-hapten response were not caused by differences in immunogenicity of the protein carrier in individual strains. All inbred strains used were tested for their ability to form antibodies against different protein carriers and against SRBC and they all gave high haemagglutination titres with only slight differences among strains.

We also have compared the antibody response to three different doses of p-aminobenzoic acid-BGG in the best and the poorest responding strains of mice, B10.LP and CBA/J, respectively. The dose dependence of the immune response was identical in both strains. The smallest dose, $3 \times 10$ µgm yielded the lowest response, $3 \times 100$ µgm and $3 \times 1$ mg provoked higher, but identical responses (Figs. 9 and 10). Thus, differences in antibody-forming capacities of the two strains are not affected by the immunizing dose in a manner that would indicate possible paralysing effects of the large antigen doses in the poorly responding strain.

To exclude the possibility that the quality of the antibody response might be merely a reflection of differences in distribution of the antibody in immunoglobulin classes and differences in the sensitivity of their serological detection, we compared the participation of immunoglobulin classes in the antihapten antibody response. Those differences that were found among the strains by radioimmunoelectrophoresis, ultracentrifugation in sucrose gradient, or 2-mercaptoethanol treatment, were only an expression of a higher or lower capability of the strains to form antibodies; no significant differences in the distribution of antibodies in the different immunoglobulin classes could be detected (Figs. 11 and 12).

To learn more about the contribution of the genotype to the regulation of immune responses, we have crossed responding and non-responding strains. All mice of the $F_1$ generation, males and females, produced antibodies in titres similar to those of the high-producing parent (Tab. 1). In back-crossing experiments, where the $F_1$ generation was crossed with the recessive parent, i.e. with an individual from a poorly responding strain, 55 % and 61 % of offspring were good producers and 45 % and 39 % responded poorly (Tab. 2). These results demonstrate that the capability to form antibodies is transmitted as a dominant factor. In no case did we find a separation of reactions to either hapten, when we used doubly-conjugated proteins containing both p-aminobenzoic and sulfanilic acid. In the $F_2$ or $B_1$ generation of parents that responded or did not respond to both haptens, the offspring always showed the reaction of one of the parents and a combined response, where the offspring responded well to one hapten but not to the other, was never found. This indicates a degree of linkage between the capability to respond to each of the haptens. It is dif-

Table 1. *Antibody responses in the $F_1$ generation to p-aminobenzoic acid and sulfanilic acid bound to bovine gamma globulin*

| Parent strains | $F_1$ | Antibody responses to p-aminobenzoic acid | Antibody responses to sulfanilic acid |
|---|---|---|---|
| C57Bl/10.LP × A | males | 13/13 | 13/13 |
| | females | 1/1 | 1/1 |
| CBA/J × C57Bl/10.ScSN | males | 22/22 | 22/22 |
| | females | 8/8 | 8/8 |
| C57Bl/10.LP × C57Bl/10.ScSN | males | 9/9 | 9/9 |
| | females | 15/15 | 15/15 |
| CBA/J × CBA/J | males | 0/8 | 0/8 |
| | females | 0/11 | 0/11 |

Table 2. *Antibody responses in the $B_1$ generation to p-aminobenzoic acid and sulfanilic acid bound to bovine gamma globulin*

| $F_1 \times P$ | $B_1$ | Antibody responses to p-aminobenzoic acid | Antibody responses to sulfanilic acid |
|---|---|---|---|
| (C57Bl/10.LP × A)$F_1$ × A | males | 8/9 | 8/9 |
| | females | 3/11 | 3/11 |
| (CBA/J × C57Bl/10.ScSN)$F_1$ × CBA/J | males | 2/6 | 2/6 |
| | females | 6/7 | 6/7 |
| (C57Bl/10.LP × C57Bl/10.ScSN)$F_1$ | males | 5/5 | 5/5 |
| × C57B1/10.LP | females | 7/7 | 7/7 |
| (CBA/J × CBA/J)$F_1$ × CBA/J | males | 0/8 | 0/8 |
| | females | 0/6 | 0/6 |

ficult to decide why a strain such as B10.LP forms anti-hapten antibodies well and another, such as CBA/J, is a poor anti-hapten producer. Since immunocompetent cells differ in their capability to proliferate, the differences found in individual inbred strains at the level of anti-hapten antibody might represent a reflection of higher or lower proliferation rates of their antibody-forming cells. To test this, we used endotoxin, a known antibody-enhancing factor. The antibody level in both inbred strains increased after endotoxin application but the difference between the two strains tested in terms of their capabilities to form anti-hapten antibody remained. Endotoxin was able to increase antibody titres in the poorly responding strain to a level that corresponds to titres reached in the responder strain without endotoxin. However, endotoxin similarly increased the antibody level in the responder strain, so that the final differences in the antibody level between the two strains remained unchanged. This suggests that the cells, making these anti-hapten antibodies have the same proliferative capacity in both strains. If the cells multiply at the same

rate, then the only explanation for the lower titres in the poorly responding strain is that the number of cells stimulated by antigen and endotoxin, and starting proliferation, is lower than in the well producing strain. This explanation is compatible with the idea that in poorly responding strains a population of cells producing a receptor site of certain structure is missing, so that the number of cells capable of reacting with the appropriate antigen is lower. If this hypothesis is correct, one would anticipate that the lack of ability to form antibodies of a certain specificity should show up as a persisting formation of only low avidity antibodies without any increase in specificity during the course of immunization due to a selection of the best fitting (high avidity) antibodies. Data along these lines are not yet available.

McDevitt and Tyan (1968) and McDevitt and Chinitz (1969) have shown that the Ir-1 genetic locus is closely bound to the strong histocompatibility gene H-2 and that it forms a part of the IX chromosom. The immune response to another antigen ($glu_{57}lys_{38}ala_5$) in mice was also shown to be genetically controlled but the corresponding gene is not dependent on the H-2 locus (Pinchuck and Maurer, 1968). Gasser (1969) has found that the capacity of inbred strains of mice to form antibodies after immunization with Ea-1 erythrocytes of wild mice is genetically controlled and that the controlling gene, Ir-2, lies in the fifth chromosom close to the genes H-3 and H-6. Experiments in which mice of DBA/1 and SJL strains were immunized against (Phe,G)-Pro–L showed that DBA/1 mice react only against the (Phe,G) portion of the antigen molecule and that this response is linked to the H-2 gene while in SJL mice the reaction is against the Pro–L part of the molecule and is not linked to the H-2 locus (Mozes et al., 1969). These results suggest that the genes responsible for the production of antibodies of different specificities are located on different chromosomes. In most cases in which linkage between the response and a histocompatibility gene was described, data are missing that would help to eliminate the possibility that the control of antibody formation might only be passive and might be due to a similarity in antigenic structure of the histocompatibility antigens and the antigen used for testing. However, the most likely explanation is that the linkage of the two genes is due to their location on the same chromosome and that they are separate units. In the case of the Ir-1, Ir-2 and H-2 gene products, indirect proof for the existence of two separate genes was obtained by McDevitt and Tyan (1968) and by Gasser (1969).

## b) The Role of the Carrier in the Genetic Control of the Antibody Response to Haptenic Determinants

The carrier for the haptenic groups of an immunogen may play a decisive role in the initiation of an antibody response. This was elegantly shown in two inbred strains of guinea-pigs that react differently when immunized with poly-L-lysine (PLL) carrier conjugated to different haptens; strain 2 becomes hypersensitive and produces a considerable amount of antibody against the hapten while strain 13 does not respond to the same hapten carrier complex (Be-

NACERRAF et al., 1963; LEVINE et al., 1963; LEVINE and BENACERRAF, 1964a). In experiments in which DNP-PLL was used as antigen, the capacity to respond was transmitted to the $F_1$ generation of responding and non-responding parents as a monogenic autosomal dominant factor. The same hapten attached to another carrier molecule (heterologous globulin, albumin) led to antibody formation in both inbred strains of guinea-pigs, which means that the capacity to respond to the hapten is fully developed in both strains. The genetic factor responsible for the reaction to the hapten conjugated to the PLL carrier does not control the direct response to the hapten but apparently controls, in some way, reactions to, or recognition of, the poly-L-lysine sequence of the carrier. According to the authors, the so-called PLL gene is responsible for this control.

Thus the carrier molecule apparently plays an important role in the reaction to an antigen. In the experiments just described the carrier, PLL, was not immunogenic in one strain of guinea-pigs. The mechanism by which the carrier intervenes in the immune response is not quite clear. According to GREEN et al. (1966) the step in which the Hp-PLL antigen is recognized as a foreign element and is changed into a form capable of inducing antibody formation is under the control of the PLL gene. There is also an alternative possibility. The gene PLL could be a structural gene controlling the antibody response against antigenic determinants whenever the carrier molecule is also a part of the determinant (PAUL et al., 1966; SISKIND et al., 1966; BEN-EFRAIM et al., 1966; BEN-EFRAIM and MAURER, 1966; GREEN et al., 1967a; LIACOPOULOS et al., 1969). But this seems most unlikely since the non-responding strain does not produce antibodies to any hapten attached to the PLL carrier and, as far as it is known, the most important role in the specificity of a determinant is played by the end group, that is by the hapten. Furthermore, the non-responding strain also does not react to any linear amino acid polymer with sequences containing lysine although there is no cross reaction among these lysine-containing antigens. Therefore, it is very likely that the PLL gene has no direct bearing on the specificity of the combining site and that it operates in some other genetically regulated step of the immune response which is common for all antigens containing lysine sequences[2].

The non-responding strain of guinea-pigs can be stimulated to form antibodies to a hapten-PLL complex, provided the complex is bound to another protein carrier. In experiments of GREEN et al. (1966, 1968), in which Hp-PLL was complexed to a negativelly charged acetylated foreign albumin (ABSA) by electrostatic forces, the non-responding guinea-pigs recognized the hapten-PLL part of the complex as a haptenic determinant. There was no difference in the anti-hapten antibody formed by the strains that responded or did not respond to PLL. Also, no differences in the fingerprints of the $F_{ab}$ fragments of these two antibodies were found (LAMM et al., 1968; LISOWSKA-BERNSTEIN, 1968). Although antibodies formed after immunization with a hapten-PLL.

[2] Some recent studies indicate that the carrier moiety is usually recognized by "T-cells" (thymus-dependent) and the hapten by "B-cells" (bone-marrow derived) and that these two cell types cooperate in the initiation of antibody formation by B cells.

ABSA complex are of the same specificity in both strains of guinea-pigs there is a marked difference in the reactivity of the two strains. The responding strain not only forms antibodies after immunization with hapten-PLL antigen but also reacts by delayed type hypersensitivity when tested with the antigen. Furthermore, the lymphocytes of these guinea-pigs can be stimulated in vitro by the hapten-PLL conjugate to synthetize DNA. On the other hand, the strain lacking the PLL gene develops only circulating antibodies against the hapten when immunized with hapten-PLL.ABSA complex but does not show either hypersensitivity reactions nor an in vitro stimulation of lymphocytes by hapten-PLL (GREEN et al., 1966, 1968).

The action of the PLL gene can not be explained simply by regulation of a degradation process. The possibility that the PLL-negative animales are incapable of handling PLL antigen could not be confirmed; on the contrary, appropriate tests showed the absence of differences in antigen breakdown in responding and non-responding animals (LEVINE and BENACERRAF, 1964b). By transfer experiments, in which bone marrow, lymph node and spleen cells from responding animals were transferred into non-responders, it was shown that the PLL gene operates on the level of the lymphocyte (FOESTER et al., 1969).

The so far clear-cut picture of the PLL gene function became a little bit more obscure when it was shown that after immunization of so-called non-responding strain with DNP-PLL, antibodies can be found with more sensitive techniques such as active anaphylaxis. Apparently these so-called non-responders are not completely devoid of the capability to form antibodies against hapten-PLL complex and the attachment of another protein carrier, like in the hapten-PLL.ABSA complex, only enhances immunogenicity (MAURER and PINCHUK, 1968). In this connection the experiments of LIACOPOULOS et al. (1969) are of special interest. These authors have concluded that the difference between reacting and non-reacting animals is in the quality of the immune response. While responding animals, after immunization with hapten-PLL complex, form antibodies of the IgM; 7S gamma$_1$ and 7S gamma$_2$ classes and develop delayed hypersensitivity, the non-responding animals form only IgM and 7S gamma$_2$ antibodies. This excludes the possibility that the PLL gene exerts an over-all control over anti-PLL reactions and moves its operation to a step in which the type of immune reaction is decided. This level could be the capacity of cells to respond to a signal since there may be a direct relationship between the potency of an antigen and the classes of immunoglobulins formed, as a result of an antigen-dependent T→B step and a "division-dependent" shift in a clone's potential for influencing the formation of different classes of immunoglobulins.

## c) Genetic Control of Tolerance Induction

Another factor that influences antibody responses is the sensitivity of an immunized animal to different antigen doses. In 1954 IPSEN described such a relationship between the antigenic dose and the induction of antibody formation

in different inbred strains of mice. The quantity of antigen needed for induction varied among the strains but was typical for individual animals of a given strain.

Doses of BSA which are usually immunogenic in some rabbits or inbred strains of mice can, in other strains, supress the immune reaction by inducing tolerance (Sang and Sobey, 1954; Sobey and Magrath, 1965, 1966), i.e. no antibody can be detected in the serum and the animals simulate the non-responders described above. In these tolerant animals responses to other protein antigens are normal. Crossing experiments between responding and "non-responding", i.e. easily suppressed, animals have shown that the easy induction of tolerance by otherwise immunogenic doses of BSA is dependent on more than one recessive gene which segregate in the population. In the easily suppressed animals a normal immune response is obtained with low doses of antigen (Hardy and Rowley, 1968).

A similar but more generalized phenomenon was described in experiments using high immunizing doses of D- or L-amino acid polymers. Polypeptides of both amino acids isomers induce antibody responses, but D-polymers do so only in a narrow range of immunizing dose. After injection of a higher dose of D-polymers, which in the case of L-polymers would be highly immunogenic, the D-polymers induce immunological tolerance (Gill et al., 1967).

This type of genetic control of antibody formation operates probably on the level of phagocytic cells or some other antigen-processing step. The non-processed antigen in excess induces tolerance and a lack of antibody response (Hardy and Rowley, 1968; Sobey et al., 1966).

## C. Theoretical Aspects of the Genetic Regulation of the Antibody Response

On the basis of the experimental material accumulated in the last few years it is evident that every antibody molecule consists of a constant region, both in the light and heavy chains, and a highly variable region which is the site of antibody specificity, the combining site. Mainly from experiments on allotypic determinants, we have learned some basic data about the genetic regulation of the constant region of the molecule. The other genetic material, gathered in studies on the genetics of the immune response, seems to describe rules that govern the genetic regulation of the structure of the variable region of both polypeptide chains. The main problem now is how to apply these two genetic regulatory systems to the mechanism of synthesis of one polypeptide chain. The variable and the constant regions of the polypeptide chain are produced as a unit (Askonas et al., 1966; Shapiro et al., 1966). As in other proteins, their synthesis starts from the N-terminal end and proceeds to the C-terminal end (Knopf et al., 1967). The difference between the synthesis of immunoglobulin polypeptide chains and the synthesis of other well studied proteins is in the variability of the initially synthetized part of the polypeptide chain.

If we accept the dogma that one cistron controls the synthesis of one polypeptide chain, than we have to assume that in antibody-producing cells a whole

population of genes with identical coding properties for the constant region exists, but that these genes differ in their coding properties for the variable region. In any classical multigene system, the gene may exchange parts of their content by crossing-over during proliferation. If the same applies to genes controlling the synthesis of immunoglobulin polypeptide chains, one would expect that crossing-over among the many genes would lead to differences

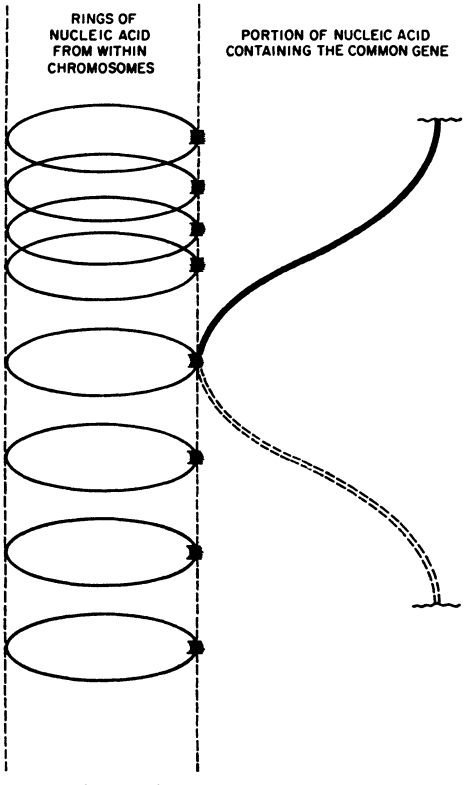


Fig. 13. Diagram of a proposed genetic mechanism that can account for variations in the amino acid sequence found in L-chains. The genetic material coding for the "variable" region of L-chains is inserted into that coding for the "common" region of amino acid sequences by a mechanism similar to the insertion of lambda-virus into a bacterial chromosome. (From DREYER and BENNET, 1965)

in segregation ratios of allotypic determinants. This does not seem to be the case, since the segregation ratio of allotypic genes follows the rule typical for classical Mendelian factors. For this reason it seems unlikely that the control of antibody specificity is realized by a series of genes with identical coding for constant and different coding for variable regions (SMITHIES, 1968). Another hypothesis postulating separate genes for variable and constant regions of the polypeptide chain is gaining in popularity. It has been suggested that two genes form one product by fusing, either on the transcription or the translation level during immunocyte differentiation (HOOD et al., 1967; HOOD and EIN, 1968; GOTTLIEB et al., 1968). No such mechanism has yet been described in any other

system but the immunoglobulin molecule seems so extraordinary in its heterogeneity that a special mechanism for its synthesis could be expected. Thus, DREYER and BENNET (1965) postulated the existence of two types of genes, one type being represented by a series of mutational variants for the variable region, the other by the gene for the constant region (Fig. 13). Representatives of both types may unite by the free movement of the constant region gene along the sequence of variable region genes to form one fused gene, responsible for the production of one polypeptide chain. According to the authors, the mechanism

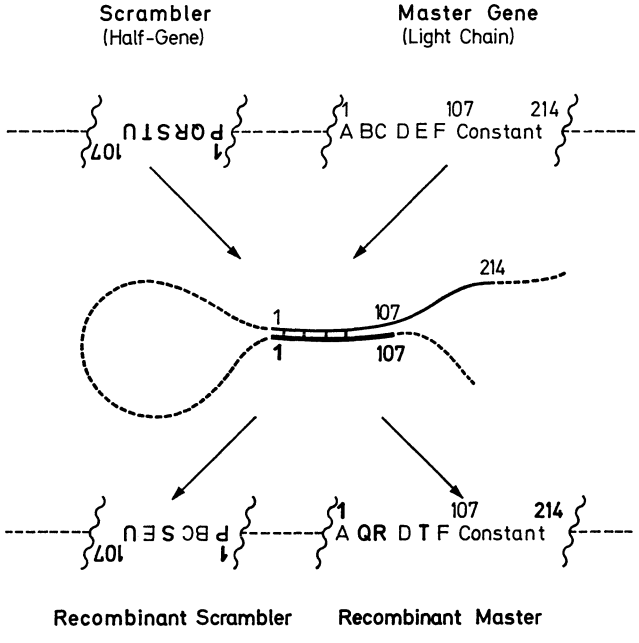


Fig. 14. One of several possible configurations that would permit a somatic chromosomal rearrangement to produce a recombinant antibody gene from the elements of an *antibody gene pair*. This particular illustrative example depicts a *master* gene for a light chain and its *scrambler* gene, an inverted duplication of the $NH_2$-terminal half of the master gene, identical to it in 101 places, but differing from it in six places. ABCDEF versus PQRSTU. The original chromosome is shown at the top of the figure, the synaptic configuration in the middle, and the rearranged chromosome below. Note the recombinant antibody gene, AQRDTF CONSTANT, differing from the nonrecombinant only in the "variable" half of the molecule. (From SMITHIES, 1967)

of fusion of the two genes is comparable to the mechanism by which lysogenic bacteriophages attach themselves to the bacterial chromosome and transform the bacteria into the lysogenic form.

Higher organisms are capable of producing antibodies with a wide range of specificity. The genetic basis for this antibody specificity remains unexplained and it is difficult to decide which of two possible mechanisms may be the likelier one. The first possibility is that for each antibody combining site there is a special gene in the germ-line of the organism. These genes would have developed during phylogeny after duplication of a progenitor gene that has under-

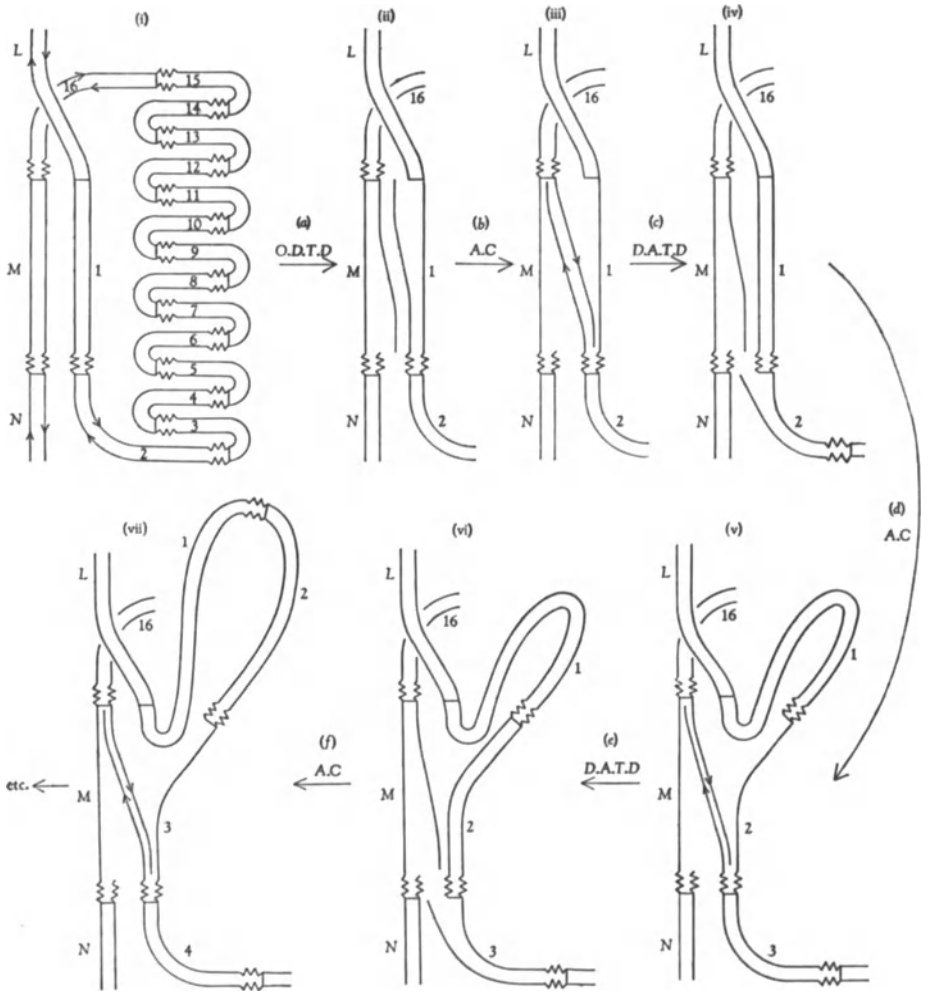


Fig. 15. Diagram to show the postulated behavior of a gene and sixteen copies of it when messenger RNA is to be synthesized. The gene and copies are arranged in a consecutive linear sequence within one DNA molecule. The copies are matched against the master gene and then extend as a lampbrush loop. The model is based on that proposed by CALLAN 1967. The lines represent nucleotide chains. Wavy lines show the position of the operator of the gene. (*A*) Annealing; (*C*) correction of mispairing to correspond with that in the chain with the descending arrow; (*D*) dissociation; (*L*,*N*) neighbouring genes to M and its copies. (*M*) master copy of gene; (*O*) breakage at operator, (*T*) breakage at terminus. (1–16), sixteen copies of gene M. It would appear, some modifications of the scheme shown in the structural genes for the antibody polypeptides, would be necessary to allow, both chains of all the other copies to be matched against on of the two copies which had taken part in an intrachromosomal cross-over. (From WHITEHOUSE, 1967)

gone point or other mutation (HOOD et al., 1967). This hypothesis runs into difficulties when one tries to place all the necessary genetic material into a given cell where it would occupy a considerable portion of the nuclear material so that not much would be left for other functions of the genome. There is also another problem. Organisms have the capacity to react also against antigens

that do not exist in nature, such as synthetic antigens or haptens, and the corresponding genes would have to survive in the genome in spite of lack of activity. This is unusual, since evolutionary pressure tends to eliminate non-active genes as unnecessary and replace them by genes with more important functions.

The other type of hypothesis assumes that the existing variability of antibodies is determined by genes that appear, by a process of somatic mutation, during ontogenic development and differentiation of lymphatic cells. The high rate of proliferation of lymphatic tissue provides a good background for the operation of such a mechanism. According to speculations by BRENNER and

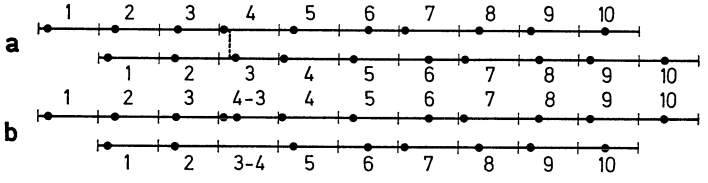


Fig. 16. Diagram indicating a single crossing-over 4–3 a hypothetical double array of ten tandem duplicated genes. The dots indicate point mutations at different positions in each gene. Other arrangements are possible. By shifting, for example, gene 6 could cross over with gene 4 etc. (*A*) before crossing-over. (*B*) after crossing-over between genes 4 and 3. (From EDELMAN and GALLY, 1967)

MILSTEIN (1966), genes may be altered during chromosome replication when enzymatically produced gaps in the DNA are incorrectly repaired, introducing new base combinations into the gene. Other authors have preferred another mechanism which postulates that the final diversity of immunoglobulin-determining genes is the result of somatic changes as a consequence of recombination among a few genes present in the genome. Different mechanisms (Figs. 14–16) have been suggested to explain this type of change so that the final diversity of antibody-coding genes may be reached (SMITHIES, 1963, 1965, 1967; BURNET, 1966; WHITEHOUSE, 1967; EDELMAN and GALLY, 1967; LENNOX and COHN, 1967). What they all have in common is that they postulate that during lymphocyte differentiation the few existing genes for immunoglobulin structure undergo somatic changes mainly by crossing-over. This results in the formation of new genes differing in structure from ancestor genes and thus capable of controlling the synthesis of new polypeptide chains. This differentiation process combined with a high mutation rate would lead to a highly heterogeneous population of cells, each cell being capable of producing different antibody structures. The antigen then merely serves a selective function, stimulating to further proliferation only those cells that possess the appropriate genetic information for a given antibody.

All hypotheses of somatic mutational origin of antibody-coding genes have to postulate exceptionally high mutation rates in lymphocytic cells, since in all other known systems somatic mutations are too rare to allow for the necessary final diversity. Randomness and rarity of these processes are a great

obstacle in explaining how it can provide the organismus with the capacity to respond to most antigenic determinants and permits it function as regularly as can be seen in inbred strains of animals. It is clear, therefore, that we have still to wait for a final explanation. Our understanding of genetic regulation in higher organisms is still deficient and it is entirely possible that the genetic regulation of antibody synthesis involves a unique, not yet described, type of control which takes advantage of a combination of both mechanisms, that is a large pre-existing set of genes for immunoglobulin molecules in the genome which may reach its final diversity by some process of somatic mutation or recombination.

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