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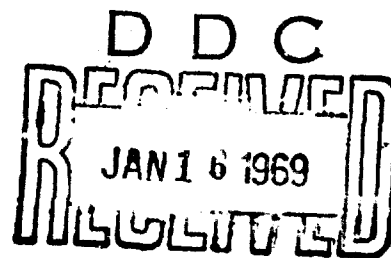
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The biochemistry of viruses.

by G. Schramm.

b) Fusion of virus and cell.

The changes morphologically visible after the entry of the virus have already been discussed. The connection of virus with certain cellular elements was demonstrated in some cases chemically. The infected tissue is fractionated in the usual manner into nuclei, mitochondria, microsomes, plasma, and the fraction to which the virus is bound is then established. Examples of this phenomenon are furnished by the herpes virus which according to Ackermann is connected to the mitochondria. Melnick has conducted similar experiments with polio virus. It is assumed that the fusion process changes not only the host, but also the penetrating particle. Many reasons support the assumption that the animal viruses also disintegrate into smaller sub-units prior to multiplication. A number of larger viruses of the vaccinia and psittacosis groups permits direct light-microscopic observation of the particles' disappearance after infection. It is only after a certain latent period that morphologically complete virus particles suddenly reappear in the cell. The smaller viruses of the influenza type at least offer the structural prerequisites for such a disintegration. Hoyle developed concepts of the details of this process (cf. influenza virus). In addition, Burnet et al. noted that an exchange of genetic factors is possible between different strains of influenza virus, similar to the interaction of phages. These results were further supported by serologic investigations by Hirst and Tamar. Double infections of chick embryos with influenza strains Melbourne and WSN produced unstable combinations and some that consolidated the serological properties of both starting strains through many passages. This indicates that in this virus the sub-units are not quite rigidly tied together, but possess a certain ability to disassociate. Additional evidence of a change in penetrating virus particles derives from the course of reproduction in relation to time. The phenomenon of the eclipse is found in all of the cases examined to date, in which the virus activity has diminished to zero or to an infinitesimal residue.

A special method that prevents secondary infections after the disintegration of the initially infected cells produces a one-stage propagation curve, as in the case of bacteriophages. The latent period of strain PR 8 is about 6 hours, that of the Lee strain 8-9 hours. The rate of propagation cannot be established with the same certainty as in the case of phages, since the amount of virus actually entering the cell is not precisely known. It is 63-fold for strain PR 8, 36-fold for Lee. A similar course of the infective process was observed by Schaffer and Hand on the chorio-allantoic membrane of chick embryos in connection with the

virus of fowl plague. After infection, virus particles that had not penetrated were removed with antiserum. After about 4 hours virus activity had risen sharply by about 3 decimal powers (Fig. 28). This jump in activity is explained best by assuming that previously masked viruses are suddenly transformed into active particles. After the expiration of the latent period the first stage may be followed by a second steep rise in viral substance contained in the egg fluid, which may be measured by HA titer (see Fig. 29). When dealing with the larger viruses, the latent period may also be determined microscopically. Table 11 reflects a compilation of observed times, extracted from D.J. Bauer's tabulation. The expulsion of mature virus particles after the latent time does not necessarily cause the lysis of the cell, as in the case of bacteria. As will be discussed in more detail in connection with influenza virus, the mature particles may be cut off from the cell wall in the form of protrusions, whereby the cell itself is preserved. Tests by Schlesinger as well as Dulbecco and Vogt indicate that infected cells are able to expulse virus several times in succession.

The inactive preliminary stages of the virus formed during the latent period were convincingly demonstrated in connection with the animal viruses. The complement fixation test of influenza virus reveals a virus-specific S antigen. The substance has a lower molecular weight than the virus proper and may be separated from the latter by fractionated centrifugation. The complement-fixing antigen invariably appears when infection is carried out with active virus, never in the case of inactive virus. Prior to the formation of mature virus, additional particles are found that already possess hemagglutinating properties, but are not yet infectious. The situation is similar in fowl plague, where the complement-fixing antigen appears first in the infected cells, then the hemagglutinating component and finally the mature virus. (See fowl plague for the chemical and physical properties of this intermediary form).

The influence of the animal viruses on the metabolism is not as easily examined as that of phages. Several authors have treated the effect of viruses on the respiration of more highly organized cells. As an example, the investigations of Caspersson and Thorsson are pointed out. The O_2 consumption in the first half of the latent period is not influenced significantly, but it rises distinctly before infectious virus is demonstrable. The question, whether the virus causes only a reversal of metabolism or whether additional processes are triggered, cannot be decided unequivocally.

Various observations exist of the effect of viruses on enzymatic metabolism, without leading to an integrated concept, however. Inhibitions as well as activations have been observed. Thus, xanthinoxydase is increased in the mouse brain after infection with neurotropic viruses. Theiler-virus evokes an inhibition of glucose up to 85% in a homogenate of brain tissue, which is neutralized by addition of diphosphoric pyridine nucleotide. Since the viruses are dependent in their multiplication on an unimpaired metabolic system of the cells, it is understandable that a lower virus propagation or increased resistance is noted when the animals are in a state of malnutrition. S. Kalter, particularly, stresses this.

2. Viruses of encephalitis.

Pending further clarification, a series of viruses are consolidated under this group designation which have failed to reveal immunological or serological relationships, but are similar morphologically and show a number of common biological properties. They are easily transmitted by insects and show good reproduction on chick embryo tissue. The following viruses are involved:

a) American equine encephalitis, western strain;
b) American equine encephalitis, eastern strain;
c) Venezuelan equine encephalitis;
d) Japanese B encephalitis and its related strains.

a) Viruses of American equine encephalitis.

Biological action. Two strains of American equine encephalitis are recognized: The western equine encephalitis, WEE, and the eastern equine encephalitis, EEE. The viruses are endemic in the USA in horses and birds, and may be transmitted to man, causing occasional small epidemics. Natural transmission is by insects. In this case the insects not only act as vectors, but the virus multiplies in them: EEE in Aedes solicitans and WEE in Aedes aegypti. In addition, other types of insects are being suspected, e.g. mites and perhaps even ticks. Various mammals and insects may be infected experimentally; the white mouse permits the most successful transmission. It is important in experimental transmission that the virus may be grown on chick embryos, representing the best starting material for chemical production. The determination of biological activity is also most convenient with the incubated chicken egg. The plaque technique on tissue cultures used by Dulbecco is also feasible. To date no serologic or immunologic relationship has been demonstrated between the two strains. A distinct differential diagnosis between these two strains and the pathogens of other encephalitides is not possible clinically; it must be made by serologic examinations. The demonstration is based on the increase in neutralizing and complement-fixing antibodies in the blood. Two samples of blood are withdrawn, one at the onset of illness, the other in the convalescent phase. A test is made to establish whether the sera are capable of neutralizing the effect of known comparative strains on the mouse. If the neutralizing effect has not increased after the illness, the existing antibodies date back to an earlier infection. If they are absent from both blood specimens, the result is doubtful, since some patients do not develop antibodies against the viruses. The demonstration of the virus proper can take place only post mortem by removal of brain material and transmission to the mouse. It must be remembered that there are encephalitides, especially post-vaccinal ones, that are not based on a viral etiology. A specific therapy of this and other encephalitides is not known; treatment with antiserum after the appearance of the first symptoms is senseless.

Eastern equine encephalitis. The EEE strain is more virulent than WEE. The mortality in the horse is high, and the symptoms in man are also

more pronounced. The disease has two phases. It begins with headaches and vomiting, the fever rises steeply after a recovery phase, at the same time cramps, paralyses and edema of the legs and face are noted. Histologically the disease expresses itself in pronounced destruction and inflammation of the central nervous system. Sixty percent of the survivors retain damages ranging from excessive sensitivity to various paralyses.

Western equine encephalitis. The WEE strain affects the same host range as the EEE strain. The symptoms in man are somewhat milder than those of EEE, they consist of head and muscle aches and various sensory disturbances, paralyses are rare. The incubation time is 5-10 days. Histologically the appearance is essentially that of meningoencephalitis, the spinal cord usually is unaffected. The largest epidemic recorded encompassed 3,000 persons, of which 8-15% died. The reproductive cycle of the virus was studied by Dulbecco on a culture of chicken fibroblasts. A latent period of about 3 hours was established. The yield of virus per cell fluctuates between a few and 150 particles.

The biochemical and morphological studies made by Beard and his school revealed that the two strains cannot be differentiated by their structure and that they possess a few conspicuous common properties which have not been found in other animal viruses so far investigated. The concept that they represent two strains of the same virus therefore seems justified. Perhaps they are related in a similar way as the cucumber viruses and TMV, which have but very few antigenic groups in common. It is conceivable that a serological relation between the two strains of American equine encephalitis could be demonstrated by the use of antisera of very high quality. It seems that such tests have not yet been conducted.

Production. The production of both strains is similar. Ten Broeck and Wyckoff were the first to identify a component in chick embryos infected with EEE which showed a sedimentation constant of 245 S in the ultracentrifuge and proved to be virus-active. Detailed research was carried out by Beard and his school. They recommend the following procedure for the production of EEE, with due consideration of the poor stability of the virus at room temperature: a 20% homogenate from chick embryo tissue is kept in Ringer solution for 72-96 hours. This lengthy extraction is necessary for the destruction of normal protein at 78.7 S, since subsequent treatment is considerably limited otherwise. Fresh extracts hardly permit the separation of the virus protein from the normal component, since the latter's concentration is three times as high. Additional work proceeds in the cold. The crude components are removed in an ordinary centrifuge and the extract is filtered through celite (a form of siliceous earth). The virus is then spun out by centrifugation for 30 minutes at 20,000 RPM (30,000 g). The sediment is reabsorbed in Ringer solution and the impurities are removed by 15 minutes at 15,000 RPM. This process is repeated twice more, until finally a solution is obtained which remains homogeneous in the ultracentrifuge and from which the normal protein mentioned above has completely disappeared. The WEE virus may be prepared in the same manner. The identity of the isolated protein with

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the virus is established by the fact that 1. such a protein cannot be isolated from healthy embryos after identical treatment, 2. virus activity is parallel to the enrichment of the protein, 3. the protein is highly active. The 50% end point is located at $10^{-13.2}$ g, corresponding to about 250 molecules. Serological tests for purity apparently have not been carried out to date.

Size and shape. On electron-microscope pictures the strains of equine encephalitis reveal a uniform spherical and disk-shaped form with a diameter of about 40 millimicrons. The nucleus seems more dense than the marginal zone. The contrast of irradiated pictures is considerably improved by the use of $CaCl_2$ (Fig. 55). The purified virus of the EEE strain sediments with a distinct band corresponding to 265 S at a concentration of about 0.4%. The sedimentation constant of WEE is $s_{20}= 273$ S, thus being identical with that of EEE within the margins of error. The frictional ratio according to measurements of viscosity is $f/f_0= 2.3$. The partial specific volume was established at 0.839 in the pyknometer. These two values yield a molecular weight of $152 \cdot 10^6$. The high frictional coefficient does not compare favorably with the spherical form observed through the electron microscope; this strong deviation from 1 cannot be explained solely by hydration. It must be pointed out that the frictional coefficient of fowl plague found in our laboratory was also very high. However, after the preparation had been purified electrophoretically, a much lower value was obtained for the frictional ratio, corresponding to the electron-microscopic appearance of the virus. It is possible that the high viscosity of the viruses of equine encephalitis is also due to impurities. The electron-optical diameter of 40 millimicrons would correspond to a molecular weight of $24 \cdot 10^6$.

Chemical action. The viruses of American equine encephalitis are the only animal viruses in which no DNA, but only RNA has been demonstrated; they occupy a special place in this respect. The entire nucleic acid phosphorus is present in the form of RNA, amounting to 10% of the entire virus. The high concentration of lipoid is also noteworthy. The viruses consist of a complex of 46% ribonucleoproteide and 44% lipoid. This, in turn, contains 64% phosphorus lipoid, 25% cholesterine and 15% fatty acids. The high lipoid content also explains the relatively large specific volume.

The virus is considerably more stable in Ringer solution than in NaCl solution. When the salt is removed by dialysis the virus becomes inactive. The virus is stable between pH 3 and 5 and between 7 and 10. At pH 5.5 the virus rapidly loses its activity. This inactivation is not strictly an effect of pH, but is dependent also upon a factor occurring in normal and diseased embryos. Perhaps an enzyme with a pH optimum at 5.5 is involved. Tests with the ultracentrifuge show that the virus protein remains constant after storage for 3-4 days at 5° and pH 7-8.5. Above pH 8.5 it decomposes into smaller, non-sedimentable components, whereby the rate of decomposition increases with pH. Below pH 7 the protein tends toward aggregation and finally becomes insoluble. The pH stability of the protein and that of virus activity coincide.

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In UV the virus shows an absorption band at 260 millimicrons. However, extinction is not attributable solely to the concentration of nucleic acid, since part of the light diffusion is surely deceptive, according to experience gained in connection with TMV. The virus is inactivated by ultraviolet light, although the homogeneity and sedimentation constants of the protein remain unchanged.

The viruses of equine encephalitis tolerate treatment with ether and with merthiolate 1:500. These reagents may therefore be used as preservatives. The viruses are also relatively preservable in 50% glycerol between pH 7.4 and 7.5. The viruses may also be maintained at a constant level by freeze-drying. Of particular interest is the reaction of the purified EEE virus with formol, since the virus, inactivated in this manner, is used in the immunization of horses and also, to a lesser extent, of particularly endangered persons, e.g. laboratory personnel. At formol concentrations below 0.01 m the protein remains in solution, but loses homogeneity. Inactivation at this level is not always complete even after two weeks. At concentrations above 0.02 m, inactivation is complete after about 4 days; the protein becomes very inhomogeneous, however, and ultimately remains insoluble. The virus gradually becomes insoluble also by formol treatment in crude extraction.

The most successful immunization was carried out with soluble protein, obtained by treatment with 0.01 m formol. A similar process permits the production of a good vaccine against Venezuelan equine encephalitis. According to Ten Broeck, inactivation of WEE without loss of antigenic activity is possible also with mustard gas. Various data exist on the efficacy of formol vaccines in man. They produce a solid immunity, for neutralizing antibodies were demonstrated even 2 years later, although complement fixation was absent. Antibodies are found also in the cerebrospinal fluid of rabbits. Accordingly, they are able to penetrate the liquor and assure protection against intracerebral infections. This observation may not be generalized, however; thus no protective effect against intracerebral infections can be induced in guinea pigs.

The viruses of American equine encephalitis have served extensively as models for general immunological studies (Olitzky, Sabin).

b) The virus of Venezuelan equine encephalitis.

The virus of Venezuelan equine encephalitis, just as the strains previously discussed, occurs principally in horses and mules in Central and South America, but infections of man are also known. Little is known about its morphology.