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THE BIOLOGY OF VIRUSES
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(Courtesy of R. W. Home. Reprinted with permission. Copyright © 1962 by Scientific American Inc. All rights reserved)

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IN writing this little book on viruses, I have departed somewhat from the traditional method of approach. Instead of dividing the book into subjects such as purification of viruses, methods of spread, morphology and ultrastructure of the virus particles etc., I have selected a number of viruses affecting plants and animals and described their 'life-history' or 'biography' if one can use such terms in reference to viruses. Each virus, or group of viruses, has been deliberately chosen because their study has resulted in a discovery of some importance or has led to the development of new techniques which have increased our knowledge of the subject. In so doing my hope has been to preserve something of the romance of scientific research which is so often bogged down in too much technical detail.

I am most grateful to Professor Michael Abercrombie F.R.S. for his continued interest and advice during the preparation of the book and also to Professor Allan Downie F.R.S. who kindly read and commented upon the section dealing with the viruses affecting man and the higher animals. However, any errors of omission or commission are my responsibility alone.

I am also indebted to those friends who kindly lent me photographic prints for the illustrations; the names of these authors are given in the list of figures.

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Frontispiece A diagrammatic representation of the sizes and structure of a number of viruses. A micron, used as a measuring stick, is a thousandth of a millimeter; it is enlarged 100,000 times. The five viruses with polyhedral structures possess cubic symmetry. The tobacco mosaic virus and the internal components of influenza and mumps virus have helical symmetry. The remaining viruses exhibit complex symmetry.
ALTHOUGH virus diseases, as typified by smallpox and poliomyelitis, have been known to exist centuries before Christ, and paintings of virus-infected tulips, dating from the time of Rembrandt, are common enough, the first serious study of a plant virus is only of comparatively recent date. In 1886 Mayer, a German botanist working in Holland, studied a disease of the tobacco plant to which he gave the name of 'tobacco mosaic' because of a fancied pattern on the mottled leaves. Nowadays, the term 'mosaic' is used to describe many plant virus diseases which cause a similar mottling of the leaves (Fig. 1). Mayer rubbed the sap from mosaic plants onto the leaves of healthy tobacco plants and found that they in turn developed mosaic, thus showing that the disease was infectious; he also demonstrated that the infective agent, whatever it might be, was no longer infectious after exposure to a temperature of 90°C. A few years later, in 1892, the Russian botanist, Iwanowski, investigating the same disease, confirmed some of Mayer's findings but disagreed with others. He agreed that the disease was
infectious and that infectivity was lost by exposure to temperatures of 90°C., he strongly denied, however, Mayer's statement that the sap was no longer infectious after passage of ordinary filter paper. Indeed, in the light of the extraordinarily infectious nature of the virus it is difficult to understand how Mayer arrived at this conclusion. In order further to disprove Mayer's contention, Iwanowski passed some mosaic sap under pressure through a Pasteur-Chamberland filter candle which removed all visible organisms. He then rubbed some of this bacteriologically sterile filtrate onto healthy tobacco plants and found it to be still highly infectious. Thus, Iwanowski laid the first foundations for the study of viruses which, under the name of Virology, that word of doubtful parentage, has now become a major science. A few years later two German workers, Loeffler and Frosch, demonstrated that the causal agent of foot-and-mouth disease of cattle was of a similar nature. From these discoveries arose the term 'filterable, ultra-microscopic viruses', a description now long obsolete as we shall see later. Mayer and Iwanowski, however, influenced by the bacteriological advances of that time, continued to regard the causal agent as a bacterium of unusually small size, a view held by a number of workers well into the twentieth century. Indeed, Mayer's efforts to prove that a bacterium was involved were weird and wonderful and included inoculations to the tobacco plants with concoctions of pigeon manure and old cheese.

On the advent of a third worker, Beijerinck, the first step was made away from the conventional bacterial approach to the problem. By a series of careful experiments on the diffusion of the infectious agent through agar, Beijerinck came to the conclusion that it
could not be 'corpuscular' and suggested that it was a 'contagium vivum fluidum', not a very precise definition perhaps, but at least an effort to visualize an unusual agent of disease.

After these discoveries little work, surprisingly enough, seems to have been carried out on the tobacco mosaic virus. Later, from the 1920s onwards, the whole trend of plant virus work was to concentrate on the disease, and this may have been in part due to the 'small bacterium' concept of aetiology, which discouraged attempts to treat the viruses from a biochemical standpoint. During this period a considerable amount of information was accumulated on the interactions between the virus and its host plants. Contrary to earlier ideas it was found that the virus would infect a very wide range of plants outside the Solanaceae to which the tobacco plant belongs. Indeed, about a dozen families of plants are susceptible; these include beans and spinach, whilst in the Solanaceae the tomato, besides tobacco, is an important host.

A great many plant viruses depend upon plant-feeding insects for their spread from plant to plant; the relationship between some plant viruses and their insect vectors is an extremely interesting one and is dealt with in some detail in Chapters Three and Four. Curiously enough although it is the most infectious plant virus known, tobacco mosaic virus (TMV) has apparently no insect vector. Probably there may be a little incidental spread by the virus being carried mechanically on the jaws of leaf biting insects. Indeed this has been demonstrated experimentally in America by Walters who held a species of large grasshopper by the hind legs and allowed it to feed first on a mosaic tobacco plant and then on a healthy one which later became infected. In fact, it is man himself who is the chief agent of
spread of this virus; owing to its stability and high thermal inactivation point the virus remains in a viable condition in commercial brands of cigarettes and pipe tobacco, and so can be conveyed to susceptible plants by the hands of smokers. There is a story about a tobacco grower who found a much higher percentage of TMV in his crop than in that of his neighbours; finding that his workers were in the habit of chewing tobacco and of expectorating indiscriminately among his plants, he issued a ration of sterilized chewing tobacco and found that the incidence of infection immediately dropped.

In commercial tomato houses where a strain of TMV gives rise to tomato mosaic, the main method of spread is on the hands and instruments of the workers who carry the infection from one end of the house to another. The question as to whether this virus is seed-transmitted is a debatable one; the virus is undoubtedly on the outside of the seed coat since it is present in the juice of the tomato fruit, and possibly accidental contamination of the cotyledons of the seedling might give rise to a small percentage of infections. In commercial practice, however, the processing of the seeds would undoubtedly tend to remove any virus adhering to the seed coat.

Up to the year 1929 there was no method of quantitative study, or assay, of TMV or any other plant virus, but in that year, Holmes, in America, demonstrated a method analogous to the 'plating out' of bacteria. Certain plants such as Nicotiana glutinosa and certain American varieties of French bean (Phaseolus vulgaris), such as Early Golden Cluster or Pinto, react to infection with TMV with a local response only (local lesions) without a systemic mottling of the whole plant. Holmes demonstrated that there was a
relationship between the virus content of the inoculum and the number of local lesions formed on the inoculated leaves. This allows for comparative estimates of virus concentration; there are, however, many variables to be taken into account when using the local lesion method and much work has been carried out to try and make the results statistically correct. For example, it is necessary to inoculate one half of the leaf only with the experimental virus sample, the other half being inoculated with the control sample. This is because all the leaves of one plant do not react uniformly to inoculation. This method of biological assay is, of course, applicable to any plant virus provided there is a plant available which reacts to inoculation with the production of countable local lesions on the inoculated leaves.

In 1928 another step forward in the differentiation of viruses was made by Helen Purdy Beale who discovered that TMV was an active antigen. She clarified the mosaic sap and injected a few ml. at intervals into the veins of a rabbit; after a few days some blood was drawn off, allowed to stand and the serum collected. This 'antiserum', as it is called since it contains the antibodies to the TMV, will react only with TMV and its related strains. The usual technique is to mix the antiserum with the virus to be tested at different dilutions in small tubes held in a water bath with a strong light behind. The reaction between the virus and the antiserum in the form of a precipitate can then be observed accurately. Using this technique Bawden and Pirie were able to show that a relationship, hitherto unsuspected, existed between TMV and a mosaic disease of cucumber known as Cucumber Virus 3. Other uses of this technique include the detection of latent virus infections and this has been applied on a large scale to the testing of
seed potatoes and, in Holland, of tulips. A more rapid and easy serological test has been developed by Dutch workers; agar is poured into petri dishes using a metal matrix to produce several peripheral and one central cavity in the agar. Antiserum is placed in the central cavity and virus-containing preparations in the cavities towards the edge of the petri dish. After standing for two to six days, zones of specific precipitate may form in the region between the cavity containing antiserum and that containing virus. For a full account of the subject, the reader is referred to Plant Virus Serology by R. E. F. Matthews published by the Cambridge University Press.

After an animal has recovered from a virus disease the antibodies produced against the virus confer an immunity for a period, which varies according to the virus concerned, against another attack of the same virus. Plants do not produce antibodies and so would not be expected to develop an acquired immunity. The reader should be clear in his mind at this juncture that in talking of plant virus serology the antibodies are developed by the rabbit in response to the virus in the plant sap.

Nevertheless, in spite of the absence of antibodies, plants do develop an acquired immunity of a kind, which, like serology, can be used within limits to detect relationship between apparently different viruses. It was first demonstrated by McKinney in 1929 that plants infected with a strain of TMV which gave rise to a light green mosaic underwent no change in symptoms after repeated inoculations with a strain of virus causing a yellow mosaic. In 1931 Thung carried out a similar experiment with a strain of TMV causing a white mosaic against the ordinary green mottling type. The exact mechanism of this kind of cross-protection is not under-
stood; probably all the available sites of multiplication are already occupied by the first virus which holds the Tort, so to speak, against the second. The high hopes, roused by this discovery, that a reliable tool for the recognition of virus relationships was now available have not been realized, and the best that can be said for the method is that a relationship between two viruses can be presumed when there is cross-protection, but the absence of this phenomenon does not necessarily imply that the viruses are unrelated.

We have seen that TMV is infectious and can easily be transmitted by inoculation, that is rubbing with mosaic sap. Indeed, so infectious is it that the mere breaking of a leaf hair, trichome, with a contaminated instrument is sufficient eventually to infect the whole plant. The question then arises, what is the mechanism that enables the virus to spread through the whole plant? We have, first to consider the tissues in which the viruses occur. TMV, like other mosaic viruses, occurs in both parenchyma and phloem. In consequence there are presumably two types of movement, first a slow cell-to-cell migration, the virus being carried round the cell by diffusion and protoplasmic streaming, passing via the protoplasmic connexions, the plasmodesms, or possibly by holes in the cell wall by diffusion. In the second type of movement, the more rapid one in the phloem, these forces presumably play no part, but viruses have been shown to move rapidly in directions of food utilization and storage and slowly in opposite directions.

That the virus is actually moving in the phloem can be demonstrated by a simple experiment. Bennett, in the U.S.A., first showed that a virus in the raspberry could be confined to certain parts of an infected plant by destroying
the phloem connexions between the inoculated portion and other parts of the plant at the time of inoculation. Caldwell, in the United Kingdom, carried out a similar experiment on the tomato plant with TMV. A 'bridge' was made in the stem by steam, so that only the xylem vessels were left; it was found that when inoculation was made the virus remained in the half of the plant inoculated, and was unable to pass the xylem 'bridge'. Caldwell also claimed that when virus was injected into the xylem vessels, it could not escape therefrom unless the vessels were mechanically injured, whereupon the leaves developed symptoms.

All these experiments with TMV have been carried out with the sap, clarified or otherwise, from mosaic-infected plants, but the virus itself, an unknown entity, still remained concealed like a black cat in a dark cellar without an assurance that the cat was even there. It is true that in 1932 Takahashi and Rawlins observed the flow of tobacco mosaic sap by polarized light and found that the sap showed the phenomenon of double refraction or anisotropy of flow indicating the presence of rod-shaped particles—an observation since amply confirmed by many methods. During this period several attempts had been made, without success, to isolate the virus by means of chemical methods. In 1935 W. M. Stanley an American biochemist succeeded in isolating the virus; it is not necessary to give the purification method in detail but the virus was precipitated several times from the clarified infective sap with acid and 40 per cent.: saturated ammonium sulphate. The final virus suspension is opalescent and on the addition of acid and ammonium sulphate a precipitate with a characteristic satin-like sheen appears. This contains microscopic needle-like crystals or paracrystals; the virus being rod-shaped
does not form a true three dimensional crystal. In parenthesis here it is of interest to mention that needle-like inclusions, very similar to those produced by purification methods, occur in the terminal cells of leaf hairs in infected plants. Known as 'intracellular inclusions' or X-bodies, they have been shown to be crystalline aggregates of the virus.

Shortly after the isolation of TMV, Bawden and Pirie in Cambridge showed that the virus was a nucleo-protein, consisting of protein and ribonucleic acid (RNA). The proportions are 94.4 protein to 5.6 ribonucleic acid.

These discoveries, of course, inaugurated an intensive study of the chemical nature of the virus, together with much investigation of the structure and ultrastructure of the virus particle by means of electron microscopy and X-ray diffraction studies.

The general elementary composition of TMV is approximately: C, 50%; H, 7%; N, 16.7%; S, 0.2%; and P, 0.5%.

The nucleic acid of the virus was first isolated by Bawden and Pirie by a number of methods including denaturation of the virus protein by means of heat in the presence of salts, by the action of pyridine on the virus and by the action of strong acetic acid on the virus. Later Sreenivasaya and Pirie prepared the nucleic acid by dissolving away the protein in a solution of dodecyl sulphate. In 1939 the structure of the virus nucleic acid was investigated by Loring who found that it had the purine bases, adenine and guanine, and also the pyrimidines, cytosine and uracil. With the advent of paper chromatography for the analysis of nucleic acid, the composition was reinvestigated by Markham and J. D. Smith. They showed that the sugar in the nucleic acid was,
as expected, ribose, and the proportions of adenine, guanine, cytosine and uracil were found to be 1.17 : 1.05 : 0.71 : 1.06 on a molar basis, and this has been confirmed by a number of workers. For a comprehensive account of the biochemistry of TMV and other plant viruses the reader is referred to the chapter by R. Markham in Volume Two of The Viruses, editors F. M. Burnet and W. M. Stanley, published by Academic Press, New York and London.

In the account of the virus of turnip yellow mosaic, which is discussed in the next chapter, it is shown that two components are always present in plants infected with this virus. Although these two types of particles look identical, one is empty and the other contains the nucleic acid in the centre; only the latter is infectious. This discovery pinpointed the essential part played by the nucleic acid in the infection process. In 1956 Gierer and Schramm in Germany, and Fraenkel-Conrat in the U.S.A., showed that the ribonucleic acid (RNA) of the tobacco mosaic virus was capable by itself of initiating infection. This important discovery has now been extended to a number of other RNA viruses, in which the nucleic acid alone has been shown to be infectious.

As we shall see later, in discussing the ultrastructure of TMV, the outside of the virus particle consists of a protein coat, the function of which is probably the protection of the nucleic acid. When the virus particle enters a susceptible cell the first stage in the multiplication process is presumably the removal of the protein coat before the replication of the nucleic acid can take place. In the biological assay of TMV, the plants Nicotiana glutinosa or Phaseolus vulgaris, are used because they respond to inoculation by the formation of 'local lesions' on the
inoculated leaf. Now it is interesting to find that when comparative inoculations are made respectively with whole TMV and the nucleic acid alone, the lesions due to the latter appear several hours earlier than those caused by the whole virus. This suggests that the time lag is due to the fact that the whole virus particle has to denude itself of its protein coat before the process of replication can begin, whereas the RNA can initiate infection and complete its reproductive cycle without this preliminary process. It also follows from this that if the RNA and the intact virus are simultaneously inoculated into a plant there is little likelihood of the intact virus interfering with the establishment of infection by the nucleic acid since the latter would have the initial start.

The shapes of plant and animal viruses fall into two main categories; the rods and the spheres (or near spheres) and the particle of TMV is, as has been already suggested, a rod. (See Frontispiece.) The overall picture of a virus particle, derived from various methods of study, is of a thread of nucleic acid, ribonucleic acid (RNA) in plant viruses, deoxyribonucleic acid (DNA) and RNA in the animal viruses, enclosed in a protein coat consisting large numbers of identical protein sub-units (capsomeres), and in the small viruses these are the only components.

In 1956 Crick and Watson suggested that all small viruses are built up on a framework of identical protein sub-units packed together in a regular manner. Klug and Caspar point out that this means a virus particle can be constructed out of sub-units in only a limited number of ways. Determination of the symmetry gives considerable insight into the substructure of a virus particle.

In 1954, Watson showed by means of X-ray diffraction studies that the protein sub-units of the TMV particle form
a helix, and 'helix' seems to be the key word in the architecture of the rod-shaped viruses. The TMV helix was investigated again by Franklin and her co-workers in 1957, using TMV to which atoms of mercury had been attached. The results of this work suggest that there are 49 sub-units in a 69 Å length of the virus; this gives a total of 2,130 sub-units in the total length of the particle which measures 3,000 Å. A model of a particle of TMV based on the X-ray diffraction studies of Franklin and Klug is shown in Fig. 2.

When the rod of TMV is fractured or broken down by various means, it sometimes happens that a small section can be seen with a hole in the centre; the existence of this hole had already been forecast from X-ray diffraction studies. Furthermore, preparations of whole virus stained with phosphotungstic acid, and viewed on the electron microscope, show this hole as a central canal running the whole length of the rod. This central canal does not contain the RNA as might be supposed. Its presence is explained by Klug and Caspar as a rather natural consequence of the fact that the sub-units cannot taper down to a wedge narrower than molecular dimensions that will fill the space near the axis of the helical array. Apparently the RNA thread does not run through individual protein sub-units, but rather the sub-units pack together in such a way as to leave space for the RNA between them. It is thought to be only 6 per cent. of the particle and appears to be all in one piece, a single-strand polymer following the pitch of the helix. On the other hand, some recent work by Markham and his colleagues suggests some possible alternatives. High resolution electron micrographs of negatively stained TMV particles show what appears to be a second helix which fits
into the central canal mentioned above; in broken fragments of the TMV, seen in plan, the RNA seems to fit between the inner helix and the outer helix of protein sub-units.

These workers find that the dry TMV rods are left-handed helices, having 162 turns in 2,890 Å. The protein sub-units number 2,650. There are 7,900 nucleo-tides in the nucleic acid, accounting for 5.1% of the I weight of the particle.

Until recently the amount of detail visible in electron micrographs of TMV had been rather disappointing, but this has been radically changed by the new method of negative staining with phosphotungstic acid. Some very interesting pictures have recently been obtained by Nixon and Woods on repolymerized rods of TMV. These show for the first time on the electron microscope an axial periodicity of about 20-25 Å, corresponding to the pitch of the helix; the hole down the axis of the particle is also visible.

However, according to Markham and his colleagues working at Cambridge, the picture obtained by Nixon and Woods has been shown to be not the virus helix but a variant structure which the nucleic acid-free portion can assume.

In Chapter Three an account is given of the disease of cucumber known as cucumber mosaic and its aphid-transmitted virus. There is, however, another cucumber mosaic, called cucumber green mottle mosaic due to infection by two closely similar viruses known as Cucumber Viruses 3 and 4, virus 4 being a yellow mutant of virus 3. These two, which, for our purpose, can be treated as one, are totally different in character from that causing the aphid-transmitted form of cucumber mosaic. Indeed, as we have already mentioned, it was shown in
1937 by Bawden and Pirie that cucumber viruses 3 and 4 were serologically related to TMV, and this was before a common host plant was known on which cross-immunity tests could be carried out. In their physical properties viruses 3 and 4 resemble TMV in every respect; furthermore it has been shown by Rochow that there is an interference between cucumber virus 3 and TMV on the cotyledons of cucumber plants. Because of the closely similar physical properties, the marked serological relationships and the cross-protection shown, it seems fair to conclude that cucumber viruses 3 and 4, if not actual strains of TMV, are closely related to it.

There are many other strains of TMV, one, which was found by Holmes in Plantago lanceolata and called by him the 'ribgrass strain', produces a type of ringspot symptom, instead of mottling, on tobacco.

Bawden has described an interesting phenomenon concerning a strain of TMV originally found infecting cowpea (Vigna sinensis) in Nigeria. Unlike the original or type TMV, this strain was capable of becoming systemic, i.e. distributed throughout the plant, in the French bean (Phaseolus vulgaris) as well as in the tobacco plant. When grown in the bean, and inoculated to Nicotiana glutinosa, it gave rise to very small and characteristic local lesions.

However, on transfer to tobacco the virus changed its character and gave rise to much larger local lesions more characteristic of the type virus. This phenomenon is reversible and is accompanied by a definite change in the amino acid content of the protein, the most important being the presence of histidine in the virus grown in bean plants and its absence in the virus grown in tobacco. This variation in the histidine content is compensated by a
complementary variation in the lysine content of the protein. As Markham has pointed out, the most probable explanation of this phenomenon is that the cowpea virus has a high mutation rate both from the tobacco type to the bean type and back again, and that the host plant dictates the type which is predominant.
CHAPTER TWO

THE TURNIP YELLOW MOSAIC VIRUS GROUP

In the previous chapter we have discussed the virus of tobacco mosaic, probably the most intensively studied of all viruses. Among the main characteristics dealt with was its method of spread, its shape, its ultra structure and the importance of its nucleic acid for replication. In this chapter we are concerned with another plant virus, also extensively studied, which differs in several important respects from the foregoing; for the sake of comparison with TMV these differences are discussed in some detail.

Shortly after the second world war some turnip plants from the neighbourhood of Edinburgh were observed to have an unusually bright yellow mottling, so bright as to resemble some types of variegation. Sap inoculation from these plants to various members of the cruciferae such as turnip, broccoli and Chinese cabbage reproduced the same bright yellow symptoms. On Chinese cabbage, in addition to the yellow mosaic, local lesions developed on the inoculated leaves. These successful transmissions and the general appearance of the diseased plants suggested that a virus was involved to which the name turnip yellow mosaic (TYM) was given.

Search was then made for an insect vector since the disease when first noticed was obviously spreading from diseased to healthy plants. The most usual insect vector of this type of mosaic disease is one or more species of aphids
THE TURNIP YELLOW MOSAIC VIRUS GROUP

(see Chapter Three); in consequence several species of this insect, including the ubiquitous Myzus persicae Sulz., which normally feed on brassica crops, were tested. All these experiments were negative, yet the undoubted spread of the virus indicated that a vector of some kind existed. Now, among the more common of the insects which feed upon crops of the brassica tribe, and especially turnips, are several species of jumping beetles, known collectively as flea beetles, or by the farmer as the turnip 'flea'. They belong to the Family Chrysomelidae, Phyllotreta spp. Although no insects of this leaf-biting type had hitherto been recorded in Europe as vectors of plant viruses, an experiment was set up to investigate the matter. Into a small insect-proof chamber of a glasshouse was put a turnip plant infected with TYM, together with a number of young Chinese cabbage plants. About fifty flea beetles were then colonized on the infected turnip and allowed free access to all plants. After a week to ten days several of the young plants of Chinese cabbage were showing signs of yellowing, but this occurred only in those which carried the round holes in the leaves made by the feeding of the beetles. Subsequent experiments confirmed that the flea beetles were transmitting the virus.

There is one fact about the feeding apparatus of leaf-eating vectors of TYM which appears curious in the light of virus-transmission by aphids and other sap-sucking insects. In the latter case, as will be explained later, all the evidence points to the saliva as the vehicle of the virus from the insect into the plant. Yet beetles have no salivary glandsls'. A comparative investigation of the buccal end of the alimentary canal of the beetles and the aphids revealed an interesting difference. In the aphid at the end of the
oesophagus and at the anterior opening of the foregut is the sub-oesophageal valve; this allows fluid to enter the gut but precludes regurgitation. Comparable sections through the flea beetle, no easy matter to accomplish since these beetles are as difficult to cut as lead shot, showed a similar valve but placed at the other end of the foregut leaving the passage clear from foregut to oesophagus. Having no saliva the beetle is forced to regurgitate some of the contents of the foregut whilst feeding in order to help digest the tissue, and here apparently lies the secret of the virus transmission. Virus-contaminated leaf tissue, or possibly virus alone, remains viable in the foregut for a period of a week to ten days so that when the beetle is feeding on a healthy susceptible plant the virus is brought into contact with the injured leaf tissue and so enters and infects the plant. This behaviour of the virus in the insect's gut emphasizes, of course, its remarkable resistance to digestion. Indeed, it has been recovered, still viable, after passage of the gut of snails.

Since there are other types of leaf-eating insects which also either have no salivary glands or regurgitate whilst feeding, it was decided to test their ability to transmit the virus of TYM. It was found that two species of grasshopper and the common earwig (Forficulidae) were all able to act as vectors. Another type of leaf-eating insect which will feed on brassica crops is the caterpillar of the large white butterfly, Pieris brassicae L.; but this larva does not regurgitate and so cannot transmit the virus. However, application of a little ether induces regurgitation; unfortunately it also takes away the insect's appetite and it refuses to eat. Nevertheless, the virus is present in the regurgitated drop of fluid since it produces infection if it is scratched into the leaf with a needle.
THE TURNIP YELLOW MOSAIC VIRUS GROUP 19

Preliminary tests of some of the elementary properties of the virus of TYM, such as the amount of dilution it would stand, called the dilution end-point, showed that the virus occurred in high concentration in the plant, sometimes as much as 4 grammes per litre of sap. If it is desired to isolate a virus this is a very important point because it is of little use to try to purify a plant virus if it is present only in very low concentration.

The purification of TYM virus is easier than with most plant viruses and is carried out with ethyl alcohol (ethanol) and ammonium sulphate. Since the multiplication of the virus in the plant is slow, it is best to use old, long-infected plants, Chinese cabbage or turnip are the most suitable. The plants are ground up, and the sap expressed; to each litre of sap 300 ml. of 90 per cent. ethanol are added. This produces a flocculent precipitate of plant proteins which is centrifuged off, leaving a yellow and slightly opalescent supernatant fluid. To this is added half the volume of saturated ammonium sulphate in water; crystallization of the virus begins in a few minutes and is completed in about four hours. The crystals are sedimented into a pellet by centrifuging and extracted with water; they are then reprecipitated with ammonium sulphate. As a rule three or four re-crystallizations of the virus from ammonium sulphate are enough; the crystals are octahedra.

The virus dissolves readily in water and is soluble over a wide pH range; the solutions are opalescent and isotropic and have a characteristic ultraviolet light absorption.

One of the most interesting and important features of the virus of TYM is the fact that it always gives rise to two types of particles which appear to be identical, but only one of them is infectious. The way in which this was discovered
is described by R. Markham. The virus preparation, when examined at that time under the electron microscope, consisted of uniform spherical particles; we shall see, however, later that improvements in the technique of electron microscopy now reveal a difference in the particles. The phosphorus content of the virus was large, about 2.13-2.24 per cent., but varied in an inexplicable way in different preparations. However, when the virus was examined in the Spinco Model E analytical ultracentrifuge, the sedimentation diagram revealed the presence of two components. Similarly, when a quantity of the purified virus was spun in the preparative ultracentrifuge for about two and a half hours at a high speed, the solution separated sharply into two parts, the 'top' and 'bottom' components. The examination of these purified virus preparations confirmed that there are two substances, a nucleoprotein and a protein, produced by virus infection. Careful inoculation tests revealed that only the bottom component was infectious; in other words, only those particles which contained the nucleic acid (RNA) could reproduce the virus. This was an important discovery because it linked capacity to infect with the presence of the nucleic acid.

The two components form crystals which seem identical; when these are examined, however, in the ultraviolet light microscope the crystals composed of top component appear light whilst those formed from bottom component appear black because of the presence of the nucleic acid. In our discussion of the structure of the particle of TMV which is a rod the nucleic acid is thought to be interlaced between the sub-units of the particle. This cannot be the case with the particle of TYM virus which is spherical or near-
spherical, as the presence of the nucleic acid on or near the outside of the particle would introduce a difference in size, and we have seen that under the electron microscope the particles are uniform. Furthermore, diffusion measurements, X-ray crystallography and X-ray scattering have confirmed that the diameters of the hydrated particles are very close, about 280 Å.

Markham suggested that the nucleic acid was probably in the centre of the nucleoprotein, the bottom component, whilst the protein particle, top component, was empty. Studies of X-ray scattering have confirmed this, showing that the nucleoprotein is essentially solid and the protein is hollow with an internal diameter of about 75 per cent. of the external one. The new methods of contrast-staining for electron microscopy give further confirmation of this, showing numerous empty particles together with the full ones.

The interesting question arises, what is the interpretation of these two types of material which are now known to occur in other viruses besides that of TYM? An easy assumption to make is that the top component is an artefact, and it is known that the use of phosphotungstic acid (PTA) for contrast-staining in electron microscopy tends to expel the central core of some small viruses. But that of course, does not apply in the present case; the top component is always present in every plant host, and the proportion is much the same regardless of age of infection and of general cultural conditions. Matthews has studied the virus in a number of different plant hosts and he finds that there is a ratio of two nucleoprotein particles to one protein particle. Matthews suggests that the top component is the empty shell from which the nucleic acid has emerged to give rise to two new particles. This is presumably by analogy with
the empty 'doughnuts' of the bacteriophages. (See Chapter Six.)

Some experiments carried out by Jeener favour the view that the empty particles are in the nature of precursors of the nucleoprotein. He found that when diseased plants are exposed to $^{14}C\text{O}_2$, the empty particles were labelled much more rapidly than the virus nucleoprotein.

Markham considers that there is no reason to think that the top component is an artefact produced during the isolation procedure, because it may be demonstrated in preparations which have been isolated in a number of different ways. It is also possible to take healthy plant sap, add the nucleoprotein to it in a pure form and re-isolate it without any top component being formed in the process.

From the work of Markham and his colleagues it is becoming apparent that there exists a whole group of like viruses of which the virus of TYM is the type. All these viruses have several features in common—the occurrence of top and bottom components, very similar morphology, varying degrees of serological relationships—and usually have leaf-biting insects such as beetles as vectors. They do not necessarily infect the same type of host plant and, indeed, their hosts are as diverse as turnips, cowpeas, pumpkins and cocoa. As we shall see later on, empty protein shells occur commonly in some animal viruses also, such as poliovirus and one of the insect viruses.

The amino acid composition of TYM virus has been studied by several workers using paper chromatography, microbiological assay methods and ion-exchange chromatography. The amino acids in grammes for 100 gm. of protein are shown in the Table on page 23.
The nucleotide composition of the nucleic acid has been studied by Markham and J. D. Smith and is of interest because of the unusual distribution of bases.

**TABLE**

Amino acid composition of turnip yellow mosaic virus
(After R. Markham)

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>A</th>
<th>B</th>
<th>C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>5.1</td>
<td>5.4</td>
<td>5.7</td>
</tr>
<tr>
<td>Arginine</td>
<td>5.9</td>
<td>6.3</td>
<td>5.1</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>2.4</td>
<td>2.2</td>
<td>2.5</td>
</tr>
<tr>
<td>Cysteine</td>
<td>0.0</td>
<td>0.0</td>
<td>2.50</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>7.7</td>
<td>8.0</td>
<td>9.6</td>
</tr>
<tr>
<td>Glycine</td>
<td>2.5</td>
<td>3.8</td>
<td>2.9</td>
</tr>
<tr>
<td>Histidine</td>
<td>2.2</td>
<td>1.6</td>
<td>2.1</td>
</tr>
<tr>
<td>Leucine</td>
<td>8.5</td>
<td>8.6</td>
<td>10.3</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>7.4</td>
<td>7.4</td>
<td>10.8</td>
</tr>
<tr>
<td>Lysine</td>
<td>5.0</td>
<td>5.0</td>
<td>10.7</td>
</tr>
<tr>
<td>Methionine</td>
<td>2.1</td>
<td>2.1</td>
<td>2.85</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>3.5</td>
<td>3.6</td>
<td>3.8</td>
</tr>
<tr>
<td>Proline</td>
<td>9.7</td>
<td>11.8</td>
<td>10.7</td>
</tr>
<tr>
<td>Serine</td>
<td>6.3</td>
<td>6.7</td>
<td>8.1</td>
</tr>
<tr>
<td>Threonine</td>
<td>11.3</td>
<td>12.2</td>
<td>15.1</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>—</td>
<td>—</td>
<td>1.14</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>2.0</td>
<td>2.2</td>
<td>2.45</td>
</tr>
<tr>
<td>Valine</td>
<td>6.2</td>
<td>6.2</td>
<td>8.45</td>
</tr>
<tr>
<td>Amide N</td>
<td>—</td>
<td>—</td>
<td>1.25</td>
</tr>
</tbody>
</table>

A, top component; B, bottom component (Fraser and Cosentino, 1957); C, whole virus (Roberts and Ramasarma, 1952) of type strain.

These are: adenine, 23; guanine, 17; cytosine, 38; and uracil, 22 residues per 100 residues. They state that this composition is so unusual that it has defied all the attempts to fit it to any of the proposed schemes of ribonucleic acid structure.
The nucleic acid may be liberated from the virus by treatment with ethanol of more than 34 per cent. in the presence of salt at neutral pH, and in the cold. The nucleic acid itself is probably much coiled inside the virus particle. Infectious nucleic acid has been recently isolated from TYMV by Haselkorn.

There seems to be some doubt regarding the ultra-structure of the TYMV particle, and the actual number of sub-units which make up the protein coat. In earlier electron micrographs of the small spherical viruses using the conventional shadow technique it was sometimes possible, if the resolution was good, to observe that the surface of the particle was irregular and bumpy. Now, with the development of the contrast-staining technique, it is possible actually to count these 'knobs' on the surface or, in other words, the protein sub-units of the outer coat.

Klug and Caspar state that all the small viruses so far studied by X-ray methods appear to have icosahedral symmetry which implies that they consist of sixty asymmetric units. These can actually be arranged in an infinite number of slightly different models but all can be visualized as consisting of twelve identical groups of five units equivalently related by the fivefold axes of the icosahedral point group.

The contrast-staining technique has entirely changed the type of electron micrograph which can now be obtained of the small viruses especially those from plants. An electron micrograph of sowbane mosaic virus made by this method is shown in Fig. 3. It is now possible to count the protein sub-units on the face of the particle, and the number of morphological units can be found by adding some combination of the numbers twelve, twenty, thirty and sixty.
or a multiple of sixty appropriate for the particular clustering arrangement. In the spherical viruses it is almost possible to deduce from the picture of the sub-unit arrangement on the surface of the particle what is the total number of sub-units by counting the number on the edges, but knowledge of the internal detail is dependent on X-ray diffraction studies. The virus of TYM has two sub-units along the edge, with one in the middle giving a total of thirty-two. It now seems as if the combination of X-ray, electron microscopy and biochemical studies will provide the information necessary to deduce the actual arrangement of the protein sub-units in this spherical virus. For a detailed account of this subject the reader is referred to A. Klug and D. L. D. Caspar (1960) on the Structure of Small Viruses: Advances in Virus Research, 7, Academic Press, New York and London.

We have mentioned earlier that there appears to exist a group of viruses with very similar properties of which TYMV is the type. Another interesting member of this group is known as the wild cucumber mosaic virus, first isolated from a naturally infected wild plant by Freitag in 1952. It is in no sense related to cucumber mosaic virus (Chapter Three) or to cucumber viruses 3 and 4 (Chapter One), and does not in fact infect the cultivated cucumber. The virus is best grown on pumpkin (Cucurbita pepo) and can be purified by centrifugation in alternate cycles of high- and low-speed. When examined on the ultracentrifuge it behaved similarly to TYMV in having 'top' and 'bottom' components, the top component lacking RNA and being non-infectious.

The diameter of the bottom component appears to be 284 Å measured by X-ray scattering; the top component gives indication of being hollow with external and internal diameters of 280 and 210 Å respectively.
Wild cucumber mosaic virus (WCMV) is serologically related to TYMV, but there seems to be no information on its insect vector.

Some viruses which affect cowpea (Vigna sinensis) in Trinidad and Nigeria probably belong also to the TYM group. They are transmitted in the same manner by beetles and on the electron microscope the particles of the Trinidad virus are also extremely similar to those of TYM. It is not known whether 'top' and 'bottom' components of this virus exist, but the electron micrograph published by Chant shows a number of empty particles which might be indicative of a top component. It must, however, be borne in mind that the negative staining technique with phosphotungistic acid does tend to expel the nucleic acid of the particles, thus producing artificial 'empties'.

It seems likely that in the future quite a number of beetle-transmitted viruses will be found to belong to the TYM group of viruses.
CHAPTER THREE

THE APHID-TRANSMITTED PLANT VIRUSES

In the previous chapters we have dealt first with the tobacco mosaic group, viruses which are highly stable, have no insect vector and which have been extensively studied. Then came the turnip yellow mosaic group which differ greatly in their morphology and methods of transmission from the foregoing. In the present chapter we are concerned with a large and rather miscellaneous group of viruses which, nevertheless, have one point in common, they are all transmitted by aphids.

As a group, viruses of this type are rather unstable and occur in low concentration in the host plant; in consequence they are difficult to isolate and purify so that there is not much information on their morphology or chemical and physical properties.

Allard, in 1914, is generally credited with the discovery that aphids could transmit plant viruses, and he claimed to have shown that one species, which he called Macrosiphum tabaci, was the vector of tobacco mosaic virus. We have seen, however, in Chapter One that aphids do not transmit this virus, so possibly Allard was working with a strain of cucumber mosaic virus which can simulate very closely the symptoms of tobacco mosaic virus in tobacco. In 1916 Doolittle and Jagger, working independently, showed that Aphis gossypii could transmit the virus of cucumber mosaic.

The number of plant viruses recorded which have aphid
vectors is now very large and is more numerous than those spread by any other type of insect; one aphid species in particular, Myzus fersicae Sulz., is known to transmit negligently fifty separate viruses.

The host range of cucumber mosaic virus (CMV) is very wide and it is easily transmissible by sap-inoculation; it infects a great variety of garden plants, both annual and perennial, and is found also in such different hosts as Daphne, Buddleia sp., privet and possibly bananas.

In herbaceous plants the characteristic symptom is a mosaic mottle; this is particularly noticeable in cucumber and vegetable marrow plants in which the fruit also is mottled and bumpy. In tomato plants CMV may produce the disease known as 'fern leaf, in which the lamina of the leaf is reduced or absent; this is not, however, an invariable symptom, depending a good deal on temperature and the strain of virus concerned. The virus is also concerned with the production of lily mosaic, especially in Lilium longiflorum, in which the symptoms consist of faint irregular, pale green streaks on the leaves. Dahlias are also susceptible to infection, and one variety, Bishop of Llandaff, can act as a symptomless carrier of the virus; it may thus serve as an unsuspected source of contamination to other dahlia varieties in which the viruses causes a visible disease.

There have been several attempts to isolate CMV and also various conjectures as to its size and shape. The most recent was made by Tomlinson, Shepherd and Walker who cultured the virus in leaves of tobacco. The leaves were harvested three days after inoculation, since tests showed that the virus reached its maximum concentration at that time, the mid-veins were removed and the tissue homogenized at 3°C. in 0.5 M potassium
phosphate buffer (pH 7.5) containing 0.1 per cent. thio-glycolic acid. The homogenate was squeezed through a glass wool pad and n-butanol was added drop-wise and constantly stirred for a further 30 minutes during which time the chloroplasts etc. were precipitated. The precipitate was separated by centrifuging at 5,000 r.p.m. for 10 minutes and the clear amber-coloured supernatant was filtered through glass wool. The virus was sedimented by centrifuging the filtrate at 30,000 r.p.m. After ultracentrifugation the supernatant was discarded and the pellet dispersed in 0.5 ml. of 0.05 M potassium phosphate buffer at pH 7.5. After further clarification by centrifuging for 10 minutes at 5,000 r.p.m., the purified virus remained in the faintly opalescent supernatant. Examination of this under the electron microscope showed the presence of uniform particles of a mean diameter of 40 mµ.

Some earlier work on the purification of two other aphid-transmitted viruses, potato virus Y and Hyo-scyamus mosaic virus, was carried out by Bawden and Pirie. They used a combination of ultracentrifugation and precipitation with ammonium sulphate. Their final product, unlike that obtained for CMV, showed the phenomenon of double refraction or 'anisotropy of flow' which indicates the presence of rod-shaped particles. This we have encountered already in Chapter One with tobacco mosaic virus.

The aphid-transmitted viruses are the most numerous of all the plant viruses, but they will only be considered incidentally here. The main theme of this chapter is the relationship of the viruses with their aphid vectors, and this will be dealt with in some detail.

In discussing the mechanism by which biting insects transmitted the virus of turnip yellow mosaic in Chapter
Two, the existence of an oesophageal valve in the aphid was pointed out; this valve precludes regurgitation of sap and thus plays a part in the transmission of viruses by this type of insect. In the Hemiptera to which the aphids belong the mouthparts are all made on a similar plan; they consist of a proboscis, the labium, and two pairs of needle-like stylets, the mandibles and maxillae, which are enclosed in the labium. This last, which has an open groove on the dorsal surface, does not enter the plant tissue during the feeding process but acts as a protective cover for the stylets which do enter the tissue. In the act of feeding, the insect presses the tip of the labium on the leaf surface, the labium bends, becomes foreshortened and the enclosed stylets are driven into the tissue.

The inner faces of the stylets which are held together by a tongue and groove mechanism enclose two independent channels known respectively as the ejection and suction canals. Saliva is apparently pumped down the ejection canal whilst the sap is drawn up the suction canal. It was thought for some years that the saliva was pumped down into the plant and the sap sucked up by means of a pharyngeal pump situated in the head. Some recent experiments, however, have shown that, if the stylets are cut off while the insect is in the act of feeding and left embedded in the tissue, the sap continues to flow out of the cut end of the stylet; this suggests that the turgidity of the plant itself plays a part in forcing the cell sap up the stylet.

Insect-transmitted plant viruses, and indeed insect-transmitted viruses in general, can be divided roughly into two groups based on their relationship with the vector. This relationship can be purely mechanical, the mosquito vector of the rabbit myxoma virus known as the 'flying pin' is an
extreme case of this, or it can be a close biological partnership such as will be described in Chapter Four dealing with leaf hopper-borne viruses. The situation as regards the relationship of aphids with the viruses they transmit is still somewhat confused in spite of the large number of aphid-borne viruses.

In 1928 Doolittle and Walker reported that Aphis gossypii Glover could take up the virus of cucumber mosaic after a five-minute feed on a diseased plant and could infect a healthy plant during the succeeding five minutes; but if then transferred to a succession of healthy plants, it usually failed to infect any more. The writer has also carried out a number of progressive transfers of the aphid Myzus persicae Sulz. and potato virus Y and showed that usually only the first plant in the series became infected, although the periods between the transfers, twenty-four hours, were rather long. Most of the work on these lines has been done by Watson, and Watson and Roberts, who confirmed that the whole process of transmission can be completed in a few minutes. These studies led to the division of the aphid-borne viruses into two groups, the non-persistent and the persistent according to the length of the period that the insect retains its infective power. A third category was added, the semi-persistent viruses, to include those cases in which retention of virus by the aphid is relatively brief but is nevertheless considerably longer than in the case of the non-persistent viruses.

Just recently Kennedy and his co-workers have introduced terms which are less empirical and which give some indication of the location of, and route followed by, the virus in the insect. For non-persistent viruses the term style-born is suggested; persistent viruses become circulative, and those viruses which have a definite biological
relationship with their vectors are known as propagative. This type of virus is more usually associated with leafhopper vectors but there is evidence that they are also associated with aphids. That leaves the semi-persistent viruses unaccounted for, and it seems a matter of choice as to which category they should be assigned. Some authorities designate them as 'stylet-borne' whilst others, for convenience and for lack of more precise knowledge, include them in the 'circulative' viruses.

These terms suggested by Kennedy et al. seem likely to be adopted by plant virologists and so they will be used throughout this chapter.

The main facts concerning stylet-borne viruses in relation to their aphid vectors are as follows:

1. Vectors are optimally infective when they have fed only a few minutes on the infected plant.
2. Virus transmission is improved if aphid vectors are starved for a period before an infection (= acquisition) feed.
3. After acquisition-feeding infectivity is rapidly lost when the vectors feed on healthy plants.
4. Infectivity is lost much more slowly when the vectors fast after acquisition-feeding.

As a possible explanation of these facts the suggestion has been made that the viruses are inactivated by some substance produced by the aphids when feeding. If such a substance is produced, however, there is no information on where it is produced or where it comes into contact with the virus.

Bradley, a Canadian worker, has made an intensive study of the aphid-borne viruses and has done much to throw light on some of these facts. For example, he showed
that the feeding habit of the insect itself was an important factor. After a period of starving, the aphid tends to move about over the leaf surface making superficial penetrations into the epidermis. This behaviour would explain why the aphid was more efficient after starving because short stabs into the epidermis are more likely to transmit the virus than one long feed from the phloem to which the insect finally settles. Similarly, an aphid is more effective after a short time on the source of infection because it is liable again to wander here and there making several short feeds into the epidermis and getting the stylets well contaminated with virus. It is important in this context to note that most of the stylet-borne viruses are present in the epidermis.

In working with the stylet-borne potato virus Y Bradley found that the aphid Myzus persicae Sulz. most often became infective on inserting the stylets only superficially into infected tobacco plants after a period without food; the further the stylets penetrated into the plant, the less likely the aphid was to be infective. Thus the percentage of aphids that transmitted the virus decreased when the stylets were inserted into infected plants for over a minute, and none of the aphids transmitted the virus after the stylets had been inserted for longer than twenty minutes. The highest percentage of aphids transmitted potato virus Y when they were transferred to test plants immediately after a single brief feeding puncture on an infected plant. The main conclusion is that M. persicae rarely becomes infective with virus Y or transmits it after the stylets penetrate beyond the first layer of cells.

In these stylet-borne viruses where aphid-transmission may be only a matter of one or two minutes,
the relationship between virus and aphid must be in a sense mechanical because of the presence of the sub-oesophageal valve previously referred to. Any virus which is swallowed by the insect must, therefore, pass into the gut, from there it must get into the blood and so into the salivary glands before it can be ejected by way of the stylets into the host plant. All this could not take place within the short time of one or two minutes or even less, therefore we must assume that the virus is either on the outside, or inside the lumen, of the stylets.

Bradley carried out some ingenious experiments on the sterilization of the stylets of the aphid after it had been feeding on a source of potato virus Y. At first he tried formalin but as the use of this led to possible complications, he exposed the tips of the stylets to ultraviolet radiations in the 2,537 Å wave-band. This rendered the aphids non-infective but only if the stylets were exposed to irradiation; if they were enclosed in the labium infectivity was not affected. Only virus near the tips of the stylets appeared to be transmitted even after more than the tips had been inserted into affected tobacco plants.

The situation with the transmission of the stylet-borne viruses seems, however, to be more complicated than a purely mechanical contamination of the stylet tips with virus. This is shown by the varying efficiency in virus transmission by different aphid species, and by the fact that one aphid species can transmit a given virus whilst another apparently similar species cannot.

Bradley and Rideout made a careful study of the comparative transmission of potato virus Y by four aphid species that infest the potato plant. Single aphids of each species were observed with a hand-lens until each had
touched its proboscis once into the infected plant and once into the healthy plant, the whole process only taking about one minute or less. It was found that the vector efficiency of the four species varied considerably even though the conditions were as uniform as they could be made. It is possible that the manner of stylet penetration into the tissue differs with each species, and such differences may largely be responsible for vector efficiency.

"The suggestion has been made by Dutch workers that minute projections on the face of the stylets, as revealed by low power electron micrographs, might account for differences in vector efficiency by adsorbing virus, whereas this would not occur in those species lacking the projections on their stylets. Further investigation is still needed into the question of the aphid-transmission of stylet-borne plant viruses.

Incidentally, the fact that the aphids can infect potato plants with virus Y in less than a minute is of considerable economic importance. It means that insecticides would not prevent the winged aphids from bringing virus into the potato crop unless the insects were killed soon after they alighted on the plants and before they could feed. This is a different state of affairs from that pertaining to the transmission of circulative viruses, as we shall see later.

We will now discuss the situation as regards the aphid-transmission of the circulative viruses. Most of these viruses, but not quite all, cannot be transmitted by inoculation of the sap as can the stylet-borne viruses. Furthermore, their transmission by aphids is a much longer business and may take several hours as compared to minutes in the stylet-borne viruses. We must assume that there is no question of contamination of stylets, but the
virus is swallowed, passes through the sub esophageal valve into the gut, thence to the haemocoel and out again via the saliva.

With many of the circulative viruses there is a period of time after feeding on the source of virus before the aphid is able to infect a healthy plant; this is sometimes referred to as an 'incubation period' but as that implies a developmental change in the virus which, in the case of the aphid-virus relationship, is not always justified, it is better designated a 'delay in the development of infective power'. This latent period varies considerably with the different viruses, the longest recorded being that of a strawberry virus known as 'Virus 3' transmitted by the aphid Capitophorus fragariae Theob., which takes ten to nineteen days, and of the Sonchus yellow-vein virus transmitted by the aphid Amphoro-phora lactucae which takes eight to forty-six days.

The existence of this latent period at once evokes the question as to the reasons for the delay; we know that there must be some delay while the virus travels round the aphid's body but this is not enough to account for such long waiting periods. One of the interesting problems in the vector-virus relationship generally is the possibility that the virus may multiply both in the insect and in the plant, and this is discussed more fully in dealing with leafhopper vectors in the next chapter. The possibility of a plant virus multiplying in an aphid has only been seriously considered with two viruses, that of potato leafroll and that of Sonchus yellow-vein disease. Potato leafroll has been in the past, and still is on occasion, a serious trouble to the potato grower, although rigorous 'roguing' on the part of the potato inspectors has greatly reduced its incidence. The host range of the virus is rather limited and, although it does infect a
number of other Solanaceous plants, from the practical point of view its importance is confined to the potato. The symptoms consist of an inward rolling of the leaf blades which become thick and leathery owing to the accumulation of starch. In some potato varieties the leafrolling may be accompanied by the development of a purple pigment and a tendency to form aerial tubers. The parent sett is frequently unrotted in the ground.

The vectors are several species of aphids, the most important being Myzus persicae Sulz. The virus cannot be transmitted by mechanical inoculation of the sap and so mere contamination of the stylets can play no part in its transmission. There seems to be no set limit to the latent period in the aphid vector and it appears possible to reduce this period by the use of host plants, such as Physalis floridana, in which the virus may be present in higher concentration than in the potato.

The leaf roll virus is a difficult one to study; it has not been isolated nor characterized on the electron microscope. The question as to whether it multiplies inside the aphid as well as in the plant is still a controversial one and there is some evidence to support both possibilities. For example, the ability of the aphids to transmit the leafroll virus is greatly decreased by exposing them to 32°C. for three to six days. The ability to transmit more regularly did not return when the aphids were kept for a further period at 20°C. These results seem to provide no evidence that the virus multiplies in the aphid since, if it did, one would expect the insects to return to their full transmitting power at normal temperature. On the other hand, the experiments of two
Dutch workers, Stegwee and Ponson, give more substantial evidence which supports the suggestion of multiplication in the insect. They succeeded in making a series of progressive transfers of the virus by micro-injection of the aphids, no easy matter since a careless puncture of the body results in a rush of premature births. This difficulty was overcome by the use of an ingenious micro-injection apparatus which allowed a superficial inoculation to be made into one of the intersegmental membranes on the dorsal side of the insect which was anaesthetized with gaseous carbon dioxide. Using either insect juices or haemolymph from viruliferous aphids, fifteen successive aphid-to-aphid passages were carried out. the insects still retaining infectivity. This is considered to involve too great a dilution of the virus and such persistence of infection could only be sustained by multiplication of the virus in the aphids.

A second case of the apparent multiplication of a plant virus in an aphid has been described by Duffus in California. The virus in question, causing the 'yellow-vein' disease of sowthistle, Sonchus oleraceus, is transmitted by the aphid Amphorophora lactucae. As we have already mentioned the latent period of the virus in the aphid is very long and transmission is independent of the quantity of virus ingested by the insect. The shortest insect-latent period recorded was eight days at 25°C., and the longest latent period was forty-six days at 5°C. From experiments carried out on the incubation and latent periods in both the plant and the insect, it seems that a certain virus concentration must be built up in the aphid vector before transmission occurs. Aphids retain infectivity throughout their lives. The sowthistle yellow-vein virus "(SYVV) is transmitted in essentially the same manner as those plant
viruses which multiply in their leafhopper vectors. (See Chapter Four.) We see, therefore, that there are so far two aphid-borne plant viruses, those of potato leafroll and Sonchus yellow vein, which we may fairly term propagative viruses, in which a definite biological relation between virus and aphid vector has been established.

The outlook for the control of potato leafroll and other aphid-transmitted circulative viruses by a direct attack on the vector is much brighter than with the stylet-Bome viruses. Because of the comparatively long period involved in the transmission process, both contact insecticides, which will kill by contact, and systemic insecticides, which are absorbed by the plant and Kill insects feeding on it, are effective in preventing the spread of the leafroll virus from infected plants within the crop.

Besides the characteristics of the different types of viruses which we have discussed, there are one or two other interesting phenomena in the relationship of virus and aphid, one of which was partly responsible for a long unsolved puzzle of plant virology.

For years it had been thought that the insect-transmissibility of a virus was a more or less inherent and fixed property of the virus in question. It came therefore somewhat as a surprise when it was discovered that prolonged separation of a virus from its vector resulted in loss of insect-transmissibility and the development of non-vector strains of the virus. For the start of the puzzle referred to above we go back to the time of the accession of King Edward VII when a Lancashire railway worker produced a potato variety which, under the name of 'King Edward', is still one of the most popular varieties. We come
now to the year 1930 when Salaman and Le Pelley were studying the virus diseases of the potato at Cambridge. In making routine grafting experiments with different varieties, they discovered that King Edward plants were infected with a latent virus infection. This became clear when scions of this variety were grafted to other potato varieties which reacted strongly with disease symptoms. To this latent virus infection the name 'paracrinkle' was given to distinguish it from another potato disease known as 'crinkle'. Furthermore, it was discovered that all samples of the King Edward potato, including the mutant Red King, contained the paracrinkle virus. Investigation of this virus revealed that it apparently had no natural means of spread; it could not be transmitted to other potato plants by sap-inoculation or by the agency of aphids and it was never found occurring naturally in any other potato variety or plant. The puzzle to be solved then was the simple question of how did the paracrinkle virus get into the original King Edward seedling since it had no natural means of spread? For many years controversy was centre on this question and 'paracrinkle' became one of the chief arguments in favour of the heterogenesis, or spontaneous generation, of viruses. However, with the recognition of new facts and the application of an old technique, the puzzle seems now to have been solved, the credit for which goes to Kassanis working at Rothamsted Experimental Station. Reinvestigating the viruses present in the King Edward potato he found two other latent infections besides the paracrinkle virus; these were a potato virus, named virus S by Dutch workers, and another commonly occurring in carnations. There was no mystery as to how the carnation virus got into the potato because it is aphid-transmitted and
has a wide host range. The next step was made possible by the early discovery of Helen Purdy Beale that tobacco mosaic virus was a good antigen which we have discussed in Chapter One. From this discovery, of course, the whole of plant virus serology has developed and this enabled Kassanis to test the serological relationships of paracrinkle virus, potato virus S and carnation mosaic virus. He found that all three were related. Now, taking into account the new knowledge of virus mutation and the loss of insect transmissibility, the answer to the problem becomes clear. The original King Edward seedling was infected by the aphid-borne virus from carnations probably in the railwayman's own garden or allotment. Then after long sojourn in the potato and after years of vegetative propagation, the virus lost its affinity for the aphid and so its natural means of spread. The practical gains from these experiments make a fitting conclusion to the story of paracrinkle. Since some viruses fail to invade the growing-point of plants, the apical meristem may be cut off and grown in tissue culture. When large enough, the plantlets can be transferred to soil and a virus-free plant obtained. By this method Kassanis obtained a virus-free stock of King Edward potatoes which not only looked slightly different from the commercial stocks but gave an appreciably higher yield.

Sometimes a virus depends upon the presence in the plant of another aphid-transmitted virus for its transmissibility by the aphid. In other words, the virus is not aphid-borne if it is by itself in the host plant. A good example of this phenomenon is given by the virus complex which affects the tobacco plant causing the disease known as 'tobacco rosette'. There are two viruses involved in the disease, called the vein-distorting virus and the mottle virus
The former virus is not sap-inculpable but the latter is so transmitted; when together in the plant both viruses are spread. By the aphid Myzus persicae Sulz., but when separated in different plants only the vein-distorting virus can be picked up by the aphid. It is not effective to feed the aphid first on the vein-distorting virus and then on the mottle virus; they must be together in the plant for insect-transmission of both to take place. Although this seems to be a rather uncommon phenomenon, it is not unique and at least one other similar case involving two potato viruses is on record. The explanation, however, of this relationship is not known; at one time it was thought that the presence of the vein-distorting virus increased the multiplication rate of the mottle virus thus making more available to the insect, but dilution tests do not bear this out. Another possible explanation is the adsorption of one virus by the other in the plant so that they are picked up as one virus by the aphid; so far there is no evidence either way for this hypothesis.

An interesting anomaly in the aphid-transmission of cucumber mosaic virus has been described by Badami. Most plant viruses occur in different strains and he was studying a strain of CMV isolated from spinach. This virus was readily transmitted by the aphid Myzus persicae from 1946 until 1955 when it suddenly ceased to be transmitted by this species but not by several other species of aphids. Specimens of Myzus persicae collected from various localities all failed to transmit the virus so it looks as if there had been some change, possibly a slight mutation, in the virus itself. It is difficult to understand, however, why it should only affect that particular aphid species and not all
of them. Since there is much evidence that stylet-borne viruses are carried mechanically on the tips of the stylets, one might suggest that, owing to a slight mutation, the virus no longer adsorbed to the stylets.

As a further complication in the relationship of viruses and aphids there is the apparent inability of certain forms of the same aphid species to transmit a virus when other forms can do so. Polymorphism, as is well known, is widespread in the Aphididae and one species may occur as winged viviparous females, wingless viviparous females, oviparous females and winged males. This difference in virus-transmitting ability was first found by Paine and Legg in 1953; they showed that the virus of hop mosaic could be transmitted by the winged spring migrants of the hop-damson aphid Phorodon humuli (Schrank) but not by one out of 3,520 wingless females tested.

Orlob and Arny have recently shown that the circulative virus of barley yellow dwarf could be transmitted by the oviparous females of the aphid Rhopalosiphum fitchii (Sanderson) but not by several other forms of the same species. The various forms of four species of aphids have been tested by Orlob as vectors of two stylet-borne viruses, potato virus Y and a virus from cabbages. Not much difference in efficiency was noted with three of the species used but there was a difference in the case of the fourth species Aphis nasturtii Kltb., and potato virus Y. The virus was transmitted by the winged migratory forms which, during part of their life-cycle live on virus-susceptible hosts but not by the oviparous and viviparous females which spend their entire life cycle on the winter host, Rhamnus sp. This suggests that there might be a vector-form specificity related to the host range of the aphid form. It cannot be
said, however, from the evidence available whether this vector-form specificity is due to an actual inability to transmit or whether it is only a relative ineffectiveness due to feeding behaviour such as mode of leaf puncture.
CHAPTER FOUR
THE YELLOWS GROUP OF VIRUSES

This is an interesting group of viruses which produce the same general type of symptom in the host plant, although there are variations characteristic of a particular virus. In the main they are not transmissible by sap-inoculation and in consequence there is not much information on the physical properties of the viruses, though there are exceptions. The chief interest of this type of virus lies in its relationship with its insect vector; we meet here once more and in a higher stage of development the true biological transmission where the virus is dependent upon two very different kinds of organisms, a plant and an animal, for its maintenance in nature. All the yellows viruses are transmitted by a particular group of insects, the leafhoppers.

About half a dozen of the yellows type viruses will be discussed in the ensuing chapter, and they have been selected because the study of each one of them has contributed something to what is known of the important relationship between virus and vector. One of the best-known of the group is the virus causing the disease known as aster yellows and we owe much of our knowledge concerning it to an American plant virologist, L. O. Kunkel, who was a pioneer in this field.

In an aster plant affected with yellows the first symptom is a 'clearing' or yellowing of the veins of the young leaves, and this is followed by a general yellowing, or 'chlorosis', not a mottling, of the new leaves. Petals which normally contain no chlorophyll frequently become quite green.
when diseased. Another striking symptom results from the abnormal production of secondary shoots; instead of lateral buds remaining dormant, they produce long, thin, chlorotic branches. The main symptoms, then, are diffuse and well-marked chlorosis, clearing of veins, occasional one-sided or sectorial infection, upright habit of growth, malformation and increased growth in certain organs, dwarfing of other organs, dwarfing of the plant as a whole and the abnormal production of secondary shoots.

In our discussion of the aphid-transmitted viruses in the previous chapter, it was pointed out that there was evidence of a biological relationship between the 'persistent' virus of potato leafroll and its aphid-vector *Myzus persicae*. Obviously, true biological transmission must involve multiplication of the virus in both plant and insect vector, and we shall now evaluate the evidence bearing on this point. In discussing the phenomenon of biological transmission we can conveniently consider it under four heads: (1) Evidence for virus multiplication in the insect vector, (2) Transovarial transmission of virus; (3) Interaction of related viruses in the insect; (4) Effect of the virus upon the vector. It should be borne in mind, however, that the phenomena discussed in the last three sections are themselves additional evidence of virus multiplication in the insect vector.

A good deal of the early evidence of such multiplication was rather circumstantial and was open to other possible interpretations. One such experiment was carried out by Kunkel in 1937 with the aster yellows virus and its leathopper vector *Macrosteties fascifrons*. Unlike the aphids the leaf hoppers are long suffering insects and are amenable to all kinds of rough treatment such as inoculation, artificial
feeding, exposure to high and low temperatures etc. Kunkel's early experiment consisted of exposing viruliferous (= virus bearing) leafhoppers to a high temperature for varying periods of time and then transferring them to young healthy aster plants. He found that if the insects were subjected to a temperature of 36°C for eleven days or less, they still retained the power of infecting healthy asters, but the delay in the development of infective power, or the incubation period of the virus, in the insect increased with the duration of exposure to heat. After twelve days' exposure or longer, the insects lost their infectivity, but could readily be made once more viruliferous by colonizing on a fresh source of virus. Kunkel interpreted this as a gradual loss of virus by the insect during the heat treatment; the longer the exposure to heat the smaller the amount of viable virus left in the insect, and, in consequence, the longer the time necessary for this residual virus to multiply up to give an infective dose. After twelve days' exposure all the virus in the leafhopper was inactivated; the fact that the insects could re-infect themselves by feeding on a fresh source of virus shows that the heat was affecting the virus and not the vector.

Black carried out an experiment designed to measure the increase of the virus in the leafhopper during incubation. He colonized a large number of leafhoppers, uniform in size and age, upon yellowed aster plants for a given time, and then removed them to rye plants which are immune to the virus. Thus all the insects received approximately the same dose of virus. At intervals a number of the leafhoppers were removed from the rye plants, ground up into a paste, made into various dilutions and inoculated into
virus-free leafhoppers. This roundabout method is necessary, of course, because the virus is not mechanically sap-transmissible. Black found that those leafhoppers which had been longest on the rye plants contained most virus since they would withstand the highest dilutions and still produce infection via the inoculated leafhoppers, the inference being that the virus had multiplied most in those insects which had remained alive longest after the intake of virus in the first place.

More direct methods of measuring virus multiplication in the insect were developed by Maramorosch. One of these arose out of the original experiment of Merrill and TenBroeck in 1934, who demonstrated the multiplication of a virus affecting horses, that of equine encephalomyelitis, in its mosquito vector by serial inoculations from insect to insect. In 1952 Maramorosch made a similar serial passage experiment and carried the aster yellows virus serially through ten groups of leafhoppers. It was calculated that the virus would be diluted approximately to $10^{-4}$ at each passage, and that it would become less concentrated in successive passages unless it multiplied in the insect. The dilution of the original virus would have reached $10^{-40}$ in the tenth passage yet tests indicated that there was just as much virus present in this as in the first passage. This is fairly conclusive evidence that the virus had multiplied in the insect.

In 1938 Trager succeeded in cultivating the virus of equine encephalomyelitis in hanging-drop cultures of mosquito tissue, and this technique was applied by Maramorosch to the virus of aster yellows thereby eliminating the need for living plants or plant material. Aster leafhoppers were artificially infected and two days later cut into pieces. Small bits of insects were kept in
sterile conditions for ten days in drops of a suitable nutrient solution containing sugar, mineral salts, amino acids, bovine albumin, and antibiotics. Afterwards an inoculum prepared from those insect tissues was injected into virus-free aster leafhoppers by means of a special type of microsyringe. Although virus could not be recovered immediately after the insects were cut into pieces, it was recovered from these bits of tissue after ten days. These tests demonstrated that aster yellows virus completed the incubation in the tissues of the insect in the absence of plant tissues.

Two other yellows-type viruses have been proved by means of serial insect-to-insect passages to multiply in their leafhopper vectors. One of these is the wound tumor virus, so-called because a tumor forms on the roots or stems of leguminous plants affected with the virus at the site of any small wound. We shall have something to say on the morphology of this virus later in this chapter. In 1952 Black and Brakke transmitted it through seven successive passages in the leafhopper Agallia constricta. This was calculated to be a dilution of $10^{-18}$ although in actual dilution tests wound tumor virus was never recovered from insect juices at dilutions beyond $10^{-5}$.

The other virus, that of maize (= corn) stunt, was passed similarly through its insect vector Dalbulus maidis.

The second aspect of our discussion on biological transmission was the transovarial passage of viruses. The first to demonstrate this was a Japanese, Fukushi, who studied the dwarf disease of rice and its leafhopper vector, Nephotettix apicalis Motsch. He showed that the virus was transmitted from a viruliferous parent insect to the offspring, but only through the female parent. Moreover, the progeny from such a parent did not itself become
infective until after a period of nine days from the date of hatching. Fukushi also showed that the virus could be passed through six generations involving eighty-two infective leafhoppers and all derived from a single virus-bearing female without access to a further source of virus.

In 1950 Black carried out similar experiments to those of Fukushi with the virus of clover club-leaf which he has shown to be transmitted through the egg of the vector, the leafhopper *Agalliopsis novella* Say. From a pair of viruliferous leafhoppers the breeding was carried out through twenty-one generations over a period of five years. The insects were fed throughout on virus-immune lucerne plants without loss of infectivity. Black has calculated that, if multiplication of the virus is not assumed, the dilution of the original virus in the parent insect exceeded $1:2.8 \times 10^{26}$. This experiment suggests that the clover club-leaf virus could be cultivated indefinitely in the insect without the necessity for a plant host. It would be interesting to ascertain whether, under these conditions, a virus can lose its affinity for plant tissue after prolonged cultivation in the insect only, in the same way that some viruses lose their affinity for insect transmission after prolonged cultivation in plant tissue only.

We have read earlier of the rice dwarf virus studied by Fukushi and it now seems as if there was a group of rather similar viruses which attack cereals. In addition to rice dwarf there is rice stripe transmitted by *Delphacodes striatella* Fallen, and wheat striate mosaic transmitted by *D. pellucida* Fabricius. Apart from host range, their transmission through the eggs of infected mothers to a large proportion of the progeny is the most important property shared by all.
In Chapter One which deals with the virus of tobacco mosaic a type of acquired immunity in the plant was described, whereby infection with one strain of a virus precluded entrance to the plant by a related strain of the same virus. This brings us to a consideration of a possible similar interaction of viruses in the leafhopper vector. The existence of two strains of the aster yellows, induced Kunkel to investigate this problem. Since the symptoms produced by these two viruses on a wide range of plants were so similar, it was necessary first to find a host plant which would differentiate sharply between the two; such a plant is known as a 'differential host". After some search Kunkel found that Zinnia elegans and Vinca rosea, periwinkle, reacted differently to infection. In both species aster yellows virus produced thin elongated side shoots whilst California aster yellows virus produced short stubby or swollen side shoots. By the use of these plants it was thus easy enough to distinguish between the viruses, and Vinca rosea was chosen for the experiments because it was a good host for the leafhopper and because it was easily grown from cuttings which are free of the variability of plants grown from seed.

The next step was to ascertain whether Vinca rosea plants infected with aster yellows were immune to infection with California aster yellows and vice-versa; cross-infection experiments proved that this was the case. Now, since these two very similar viruses will cross-protect against each other in the plant, Kunkel argued that they might also immunize against each other in the insect as both are capable of multiplying in leafhopper cells. In order to investigate this possibility the following experiment was carried out. Three colonies, consisting of about 30 virus-
free young aster leafhoppers each, were placed on 3 healthy young aster plants in lantern globe cages. A similar colony was put on an aster plant affected by aster yellows in a fourth cage, and another similar colony on an aster plant affected by California aster yellows in a fifth cage. The colonies were kept on the plants for 2 weeks. At the end of this period one colony that had been confined on a healthy aster plant was transferred to a cage containing an aster plant affected by aster yellows. Another was transferred to a cage containing an aster plant affected by California aster yellows. The third was transferred to a cage containing a healthy aster plant; this colony was to act as a control. The colony that had been confined on an aster plant affected by aster yellows was transferred to a cage containing an aster plant affected by California aster yellows; and the colony that had been confined on an aster plant affected by California aster yellows was transferred to a plant affected by aster yellows. The colonies were kept on these plants for 2 weeks also. Then each colony was transferred daily to a series of healthy young aster plants for 4 days. The 45 aster plants on which the colonies fed during the 11-day period were kept under observation for 3 months. The results of these experiments were as follows, the virus-free leafhoppers that were allowed to feed for 2 weeks on an aster plant with aster yellows acquired and transmitted aster yellows virus, but the leafhoppers that were infective for California aster yellows virus before they were allowed to feed, during the same 2-week period, on an aster plant with aster yellows did not transmit aster yellows virus. The virus-free leafhoppers that were allowed to feed for 2 weeks on an aster plant with California aster yellows acquired and transmitted California aster yellows virus, but
FIGURE 1 A tobacco plant infected with a virus which produces a characteristic mosaic mottling on the leaves.

FIGURE 2 A model of part of a tobacco mosaic virus particle showing the protein sub-units set in a helix; some sub-units V have been removed to show the strands of ribonucleic acid (RNA).
FIGURE 3. An electron micrograph of a small plant virus, that causing sowbane mosaic, negatively stained with phospho-tungstic acid (PTA), showing how the subunits (capsomeres) of the protein coat are delineated. X 250,000
FIGURE 4 A particle of a T₂ bacteriophage which has been osmotically shocked to liberate the deoxyribonucleic acid (DNA). X 38,000
FIGURE 5 Intracellular Development Cycle of Bacteriophage T₂ (ultrathin sections)
(a) Cell before infection
(b) Nucleur breakdown immediately following injection of phage-DNA
(c) Formation of phage-DNA accumulating in the 'pool' (about ten minutes after infection)
(d) Phage-DNA condenses in particles which then become surrounded by the protein membrane of the phage
(e) Accumulation of phages before lysis of the cell.
X 20,000
leaf-hoppers infective for aster yellows virus before they were allowed to feed during the same 2 weeks on a plant with California aster yellows did not transmit California aster yellows virus.

These experiments demonstrate that the strains of aster yellows virus protect against each other in the insect vector as effectively as they do in plants. Kunkel points out that what has been proved is that leafhoppers which pick up one strain of the virus are prevented from transmitting the other; it has not been proved that a leafhopper cannot pick up both viruses.

More recently (1958), Maramorosch has carried out similar experiments to those of Kunkel on cross-protection in the insect vector. He used the virus disease of corn (maize) stunt transmitted by the leafhopper, Dalbulus maidis De L. and W., of which two strains exist, the Rio Grande (R.G.) and the Mesa Central (M.C.) respectively. The experiments of Maramorosch confirmed Kunkel's original discovery of the existence of cross-immunity in an insect vector although the behaviour of the corn stunt viruses was slightly different. Insects which acquired the two strains of the virus within a comparatively short interval of time were able to transmit either strain or both strains. When the virus-acquisition periods became longer, or the interval between these periods was lengthened, unilateral protection occurred. In other words, insects which acquired the R.G. strain first became immune to infection with the M.C. strain. However, when the M.C. strain was acquired first, it was transmitted initially, but later this was followed by R.G. transmission, despite the fourteen-day interval between acquisition of the two strains. Maramorosch suggests that the interrelationships of virus strains in vector hosts depend upon a variety of
factors such as the virulence of the virus, the length of the acquisition period, the time necessary for systemic invasion of the insect by the virus, movement of virus in the arthropod host, temperature and other factors. In fact, we know little about the mechanism responsible for strain protection in either insects or plants.

A natural corollary to the multiplication of a virus within an organism would be the production of visible disease symptoms and, indeed, the question as to how far an insect vector is affected by the virus it transmits, is one which has been often asked. Until recently the only case known of this phenomenon was the eventual destruction of the louse by the typhus rickettsiae which it transmits. Now, however, evidence is accumulating that some plant viruses which multiply in their vectors do have a deleterious effect on the latter. Although Kunkel was unable to show any difference in the length of life and breeding capabilities of viruliferous and non-viruliferous aster leafhoppers, and earlier attempts by Dobroscky had failed to show any cytological abnormalities, some work by Littau and Maramorosch suggests that the aster yellows virus does have some cytological effects on its insect vector. A difference was found in the appearance of cells of the fat body: in non-viruliferous insects the nuclei of these cells tended to be round or to have smooth contours with homogeneous cytoplasm in the unbroken cells. In viruliferous insects almost all nuclei of the fat body cells were stellate and the cytoplasm was reticulate. Many cells seemed abnormal and appeared broken in the sections. It is interesting that this apparent pathological effect should be centred in the fatbody which is the site of multiplication of several insect viruses. (see Chapter Seven.)
These histological changes do not appear to have any perceptible adverse effect upon the aster leathopper, but a case where the virus is eventually lethal to its vector has been described by Jensen. The virus causing peach yellow leafroll in California was shown to cause the premature death of its leaf hopper vector, Collddonus montanus (Van Duzee). In a series of carefully controlled experiments it was found that 12 leafhoppers which transmitted virus had a mean longevity of 38 days compared to 82 days for the non-infective controls. In another test the mean longevity of the infective and non-infective groups respectively was 24 and 51 days. In fourteen such experiments the mean longevity of 116 leafhoppers that transmitted virus was 20 days compared to 51 days for the 64 non-transmitting individuals. It might be suggested that the earlier death of the viruliferous insects is due to an effect of the altered physiology of the diseased host plant, but this seems unlikely in view of the fact that leafhoppers, which for some reason failed to pick up the virus, but nevertheless fed on the diseased host plant, had the normal span of life.

These facts concerning the intimate relationship between plant viruses and their insect vectors raise the question as to whether insects may have been the original hosts of these viruses which later became adapted to plants. Andrews points out that in considering viruses of three very different kinds, those affecting insects, vertebrates and plants, with each group insects come into the picture, and asks is it reasonable to look for the origin of viruses in insects?

Before concluding our discussion of the relationship of certain viruses with their leafhopper vectors it may be worth while to refer to the variations which occur in vector
efficiency, or, in other words, the variability in transmission by leafhoppers. The main contribution to this aspect of plant virology was made by Storey working with the streak disease of maize in E. Africa and its vector Cicadulina mbila Naude. He demonstrated the existence of two races of this insect, externally indistinguishable, which were respectively able and unable to transmit the maize virus. Storey named these races active and inactive, according to their transmitting capacity. He also showed that by the crossing of pure races the ability to transmit is inherited as a simple dominant Mendelian factor linked with sex. Storey also carried out some interesting experiments in inoculating and puncturing leafhoppers. This was probably the first record of the successful inoculation of a plant virus into an insect vector to render it infective. By this means it was possible to show that in an active infective leaf-hopper the virus was present in the contents of the rectum if the insect had fed recently on a diseased plant but not otherwise. It was also present in the general contents of the thorax, abdomen and blood, but not in the naturally voided faeces. The virus appeared in the blood before the insect developed infective power. It was also possible to render an inactive insect capable of transmitting the virus if a simple puncture was made in some part of the intestine. Inactive races could also be rendered infective by inoculation with the streak virus, although the numbers of successes were significantly less than with active races.

This is probably in no way an isolated phenomenon, but may occur with several of the leafhopper-transmitted viruses. Indeed Black has shown that something of the same sort occurs with the New York strain of potato yellow dwarf virus and the leafhopper Aceratagallia sanguinolenta.
Prov., although this case was not quite so clear-cut as the foregoing. Similarly, Watson and Sinha, working with the wheat striate mosaic virus and the vector *Delphacodes pellucida* Fabr., isolated races of the insect with varying abilities to acquire and transmit the virus.

Later on (see Chapter Twelve) we shall be discussing viruses which give rise to tumours in animals so that it will be appropriate here to mention one of the leaf-hopper-transmitted viruses which produces tumours in plants. The outstanding characteristics of the disease are the enlargement of the veins and the development of woody tumours on the roots and sometimes on the stems of infected plants. Black called it the 'wound tumour virus' because of the tendency for a tumour to form at the site of any small superficial wound. Thus, a tumour is frequently found on the stem of a potted infected plant where it comes into contact with the edge of the pot, and the large number of tumours on the roots can be accounted for by the breaking of root hairs as the roots move through the soil. Black carried out an experiment in which insect pins, 0.25 mm. in diameter, were used to make single punctures at the mid-inter-nodal points on stems of systemically infected sweet clover plants (*Melilotus alba* Desr.), a common host of the virus, and of corresponding healthy plants. From 387 punctures on infected stems 175 tumours developed, whereas no tumours developed from 505 control wounds in healthy stems.

Histological investigation by Kelly and Black of the tumours produced in the roots of sweet clover by the wound tumour virus showed that the tumours are initiated by tangential division in cells of the pericycle, opposite the primary phloem. Abnormal cell multiplication, and not
cell enlargement, is responsible for subsequent tumour development. Phloem is the first tissue to differentiate at the base of the tumour; shortly afterwards xylem differentiation follows at the periphery and this proceeds internally as radiating finger-like extensions. Growth of the tumour continues by cell division in the meristematic tissue between the xylem and phloem and between the xylem extensions.

By the use of the new techniques of gradient centrifugation and negative staining for electron microscopy with phosphotungstic acid Black and his co-workers have obtained considerable information on the size and shape of the wound tumour virus particle, and a recent paper by Bils and Hall gives further details of the structure of the virus. For their work they used highly purified virus prepared from root tumours by the technique of density gradient centrifugation (see page 118). Using the method of double shadowing for electron microscopy (see Chapter Seven) the virus particle was shown to be an icosahedron in shape and measuring about 600 A in diameter. The surface consists of sub-units about 75 A in diameter numbering four along an edge and making ninety-two in all. There is an inner core measuring about 350 A in diameter which probably consists of nucleic acid (RNA) or nucleoprotein, indicated by the increase in density with uranyl acetate staining. When the protein coat of the virus was disintegrated by removal of salt long strands, about 30 A in diameter, presumably of RNA were released. High resolution electron micrographs of potato yellow dwarf virus (PYDV) show the particles to be rather large and somewhat indeterminate in shape; an average size would be about 110 mµ in diameter.
Appears to be membrane surrounding a central core, the membrane being set with numerous projections somewhat similar to those on the influenza virus particle (Fig. 8)
CHAPTER FIVE

TOMATO SPOTTED WILT VIRUS:

TOMATO BUSHY STUNT AND SIMILAR VIRUSES:

SOIL-BORNE, MITE-TRANSMITTED AND FUNGUS-BORNE VIRUSES

The viruses discussed in this chapter form a somewhat heterogeneous collection, but all have been selected deliberately because the study of each one of them has resulted in an important contribution to our knowledge of plant viruses.

TOMATO SPOTTED WILT

The virus of tomato spotted wilt (TSWV) was first recorded in Australia in 1915; it was investigated by Samuel, Bald and Pittman in 1930 who named the disease and showed that the virus was transmitted by one or more species of thrips, Frankliniella insularis Franklin, the black carnation thrips and Thrips tabaci Lind, the onion thrips. In 1931 it was recorded for the first time in Europe by Smith working in Cambridge who discovered it in an ornamental plant Solanum capsicata, on which it produced bizarre patterns of concentric rings; the chief vector in Europe is Thrips tabaci. At the time of writing TSWV is the only virus certainly known to be transmitted by thrips, although it seems likely that other plant viruses with similar vectors must exist.

The virus is now present in many parts of the world and this is partly due to its almost unlimited host range
TOMATO SPOTTED WILT VIRUS

whereby it is liable to be imported into other countries in the tubers of such plants as dahlias which used to be commonly infected. The name spotted wilt is rather an unfortunate one, bronzy wilt would be more descriptive since the main symptom on tomato is a striking metallic bronzing of the leaves. Abroad it has been known by various other names such as Pineapple yellow-spot or Kromnek or Kat River disease in Africa.

The virus, when extracted from the plant, is very unstable and loses infectivity in 4-5 hr. at room temperature. This rapid inactivation is probably due to oxidation effects, and the longevity of the virus can be increased by keeping it at low temperatures or by the addition to the sap of certain reducing agents such as sodium sulphite. The later is useful when making sap-inoculation from certain plants, particularly chrysanthemums, when these are being tested for the presence of the virus.

Mainly because of its instability not much is known of the size and shape of the virus particle but it is thought to be an oblate ellipsoid varying from 90 to about 120 mµ in the greater diameter.

There are one or two interesting facts connected with the transmission of the virus by thrips; the delay in the development of infective power within the insect is very long. In other words it may take as long as nine days after feeding on a source of virus before the thrips becomes infective to a healthy plant. This long delay has been quoted as evidence that the virus multiplies within the insect, but other evidence is needed before this conclusion can be accepted, especially in view of the fact that the virus is easily transmitted by sap-inoculation.

Another interesting fact in the relationship between
virus and thrips is the inability of the adult thrips to pick up the virus de novo. It is necessary for the insect to feed in the larval stage upon an infected plant for it to become viruliferous; the adult arising from an infective larva is then itself infective. The reason for this phenomenon is not certainly known, although Bawden has suggested that the gut wall of the adult insect may be more impermeable to virus than that of the larva. On this assumption virus picked up during the larval stage of the insect would pass through the gut wall and circulate in the blood, where it remains for the rest of the insect's life, thus allowing the adult to transmit the virus but not to acquire it. Histological examination of the gut walls of larva and adult respectively revealed no apparent difference between the two.

Best has carried out a number of interesting experiments with TSW virus which he considers prove the artificial production of recombinant strains or new hybrids and offers a considerable amount of evidence in support of his claims. He used two naturally occurring strains A and E of TSWV; these were diluted to the highest dilution which would give infection on 40 or more half-leaf replicates distributed over 5 plants. Usually only one local lesion was produced, called single lesion at limit dilution, and this ensured so far as possible that only one strain of virus was concerned in the production of the lesion. The two virus strains, after being purified by this technique, were then inoculated as follows. Twenty-five tomato plants, 25 plants of Nicotiana glutinosa L. and 25 plants of Nicotiana tabacum L. were each arranged in 5 random blocks of 5 plants per block. The inocula were applied to the upper surface of the lower leaves (3 or 4 leaves per plant) with a ground glass spatula; the leaves were dusted with a
diatomaceous earth known as Celite before inoculation to increase the number of points of entry of the virus and excess inoculum was washed off. Strain A alone was applied to one block of 5 plants of each of the three indicator species, strain E alone to a second lot, a mixture of strains A and E with A in excess to a third lot, and a mixture of strains A and E with E in excess to a fourth lot; the fifth block of each species was used as control, giving 15 control plants for the 60 plants inoculated.

From these inoculations three strains were isolated and named R₁, R₂ and R₃; these were subjected to the single lesion at limit dilution procedure and yielded products which bred true, suggesting that they were definite strains of the virus. If a capital letter is used to denote the presence of a particular determinant and a small letter to denote its absence, then the strains correspond to the following genetic formulas based on seven chosen markers: Parent A, ABcdefg; Parent E, abCDEfg; Recombinant R₁, aBCdefg; Recombinant R₂, abCdEfG; Recombinant R₃, abCdeFg.

The parent and recombinant strains all 'breed true' when biologically purified by single lesion isolation at limit dilution, and the formulas remain the same after purification in vitro by differential centrifugation.

**TOMATO BUSHY STUNT**

The disease caused by this virus (TBSV) was described for the first time by the writer in 1935. It appeared in one or two commercial tomato houses in the west of Ireland and on one plant from Northern Ireland. After a year or two, outside of laboratories, it completely disappeared and there has only been one record of its natural occurrence in the
British Isles during the last twenty-five years. It has, however, been recorded on two occasions in Italy.

The chief symptoms on young tomato plants caused by TBSV are cessation of upward growth and yellowing and purplish coloration of the leaves. In very young plants the lower leaves may wilt and the plant frequently dies; occasionally a gross necrotic lesion may develop in the stem, causing the plant to fall over. In older plants there is almost complete cessation of growth, the youngest leaves frequently become pale yellow in colour and twist over, sometimes being completely reversed. Secondary shoots develop and these produce the bushy or resetted plant from which the disease gets its name of 'bushy stunt'. The fruit does not always show symptoms, but when these are present they consist of a characteristic mottling or blotching of pale spots or ring-like marks on a darker background. Unlike the virus of tomato spotted wilt TBSV has a restricted host range. It does, however, produce a very distinctive crinkling and blistering of the leaves of Datura stramonium, together with a marked yellow and green mottle. This plant is not only a good differential host but acts as a fruitful source of virus; another good differential host is the cowpea, Vigna sinensis. When the first leaves of this plant are inoculated characteristic local lesions develop three to four days after inoculation; these lesions, at first pale, rapidly turn red at the edges and increase in size while still retaining their deep red edge and pale centre. In cowpea the virus is confined to the inoculated leaves and there is no systemic spread in the plant.

Tomato bushy stunt virus is easily transmissible by sap-inoculation but no insect vector is known. There is some evidence that the virus is soil-borne, and this
FIGURE 6 Photomicrograph of a cell from the midgut of a 'tent' caterpillar infected with a cytoplasmic polyhedrosis; note the polyhedral crystals in the cytoplasm but not in the nucleus (top right). X 1000

FIGURE 7 Ultrathin sections of an insect virus, the Tipula iridescent virus; note the five- and six-sided contour of the virus. X 25,000
FIGURE 8 Influenza virus particles negatively stained with phosphotungstic acid; note the regularly spaced projections round the particles. X 350,000
FIGURE 9 Two particles of Influenza A$_2$ virus emerging in close proximity from the surface of a rhesus monkey kidney cell; note projections on their surfaces.  X 165,000

FIGURE 10 Single filamentous particle of Influenza A$_2$ virus emerging from the surface of a rhesus monkey kidney cell.  X 24,000
FIGURE: 11(a) A model of an icosahedron shadowed by two light sources and oriented so that an apex of the hexagonal contour points directly to each light source. This throws two shadows; one is four-sided and pointed, and the other is five-sided with a blunt end.

(b) A particle of the Tipula iridescent virus frozen-dried and shadowed in the same way; the similarity between the shadows thrown is evident.  X 105,000
type of transmission is dealt with later in this chapter. TBSV has the distinction of being the first virus to be crystallized in the form of three-dimensional crystals which are usually rhombic dodecahedra. This was achieved in 1938 by Bawden and Pirie who first clarified the sap by heating to 60°C and then precipitated the virus with ammonium sulphate. The virus is deposited on the addition of salt as an amorphous precipitate which is more soluble in the cold than at room temperature. This is fortunate because the solution can then be centrifuged in a refrigerated centrifuge to remove impurities. On further standing the crystalline form of the virus separates out as rhombic dodecahedra.

The virus contains 15 per cent. of ribonucleic acid and the relative proportions of adenine, guanine, cytosine and uracil are 25, 28, 21 and 25 on a molar basis.

Besides being the first spherical virus to be crystallized TBSV was the first on which definite evidence on sub-structure was obtained. The early X-ray studies of Carlisle and Dornberger in 1948 showed that the unit cell, the smallest repeating unit, was cubic in shape with a cell side of 386 Å. In 1956 Caspar obtained X-ray photographs that proved the symmetry to be cubic. The crystal lattice is body-centred cubic, that is, the unit cell contains virus particles at the corners of the unit cube and one in the centre. Each particle is thus surrounded by eight nearest neighbours.

Klug and Caspar point out that the symmetry of the crystal lattice is tetrahedral, which implies that the individual virus particles have at least this symmetry and must therefore be made up of twelve, or a multiple of twelve, identical sub-units.
SOIL-TRANSMITTED VIRUSES

In the early days of plant virus research the idea that these agents could be transmitted through the soil was readily accepted. This is understandable because the pioneer Beijerinck had shown in 1898 that tobacco seedlings contracted tobacco mosaic when grown in soil collected some months previously from around the roots of an infected tobacco plant. But for many years after this there seemed to be a fixed idea among plant virologists that the only important method of spread was by means of insect vectors, and that any alternative possibilities of dissemination through the soil by an entirely different type of organism were of negligible importance. Now, however, as we shall see, the investigation of the soil-borne viruses is becoming an important part of plant virology. It will be necessary, before discussing this aspect, to be quite clear as to the exact meaning of the term 'soil-borne'. For a definition we cannot do better than quote the following from Harrison—'a soil-borne virus is defined as a virus with an underground natural method of spread which does not depend simply on contact between tissues of infected and healthy plants'. Under this definition tobacco mosaic virus and potato virus X, which spread by contact between infected plant debris, and by root contact respectively, are excluded.

An early-discovered example of a soil-borne virus is that of wheat mosaic which was studied by Webb in 1927 and 1928, and by McKinney and co-workers in 1925; another example is that of the suspected virus causing the mosaic disease of oats also studied by McKinney in 1946. Attempts have been made recently to discover something of
the mechanism involved in the spread of these two viruses. Certain chemicals such as formaldehyde, chloropicrin, carbon disulphide and ethyl alcohol easily rendered infective soils non-infectious; but toluene had no effect. When plants were grown in autoclaved soil, to which roots from naturally infected plants from the field had been added, they became infected. This did not occur, however, when the soil was inoculated with virus-infected sap or leaves, or with roots or leaves from plants mechanically infected. Much the same result was obtained with oat mosaic, so the conclusion must be that there is something, a hypothetical vector of some kind, which is present in naturally infested soils and closely associated with the roots. McKinney showed that, whatever this something is, it must pass through a 250-mesh sieve but, according to Webb, not through cheesecloth or filter paper. Several workers have searched unsuccessfully for this agent so that for the present the problem remains unsolved.

The large number of soil-borne viruses, which are now known, can be assembled into groups the members of which have rather similar characteristics. In addition to the viruses affecting cereals, which we have already discussed, there are the tobacco necrosis viruses and similar agents, the grapevine viruses, and the rather large ringspot group of viruses, so called because of the tendency of the symptoms on the leaves to take the form of rings with a central spot. Besides these, one or two viruses, which differ in various ways, fall into categories of their own, such as the viruses of tobacco stunt, tobacco rattle and peach rosette mosaic.

A notable contribution to our knowledge of the soil-transmission of plant viruses was made in 1958 by Hewitt and his colleagues working with a disease of the grapevine
known as 'fanleaf. They were the first to incriminate as a vector a soil-living organism, and showed the virus to be transmitted by a nematode worm, a large migratory dagger nematode (Xiphinema index, Thorne and Allen) with a long mouth spear. Eelworms of this type are phytophagous and feed in the roots of a great variety of plants.

The first evidence that this nematode could transmit the virus was obtained by growing healthy and diseased plants in the same pot and by adding X. index to some of the pots. The fanleaf disease developed in the healthy plants only when the nematode was present. The disease also developed when the nematodes were collected from the root environment of diseased plants and added to the soil containing the healthy plants. On the other hand no disease developed if the nematodes used were collected from the roots of healthy fig trees.

Following upon this work several other soil-transmitted viruses have been found to have nematode vectors, notably those belonging to the ringspot group, of which Arabis mosaic virus (AMV) is one of the most interesting. This virus which was first observed in a plant of Arabis hirsuta growing in the glasshouses at Cambridge, instead of being a laboratory curiosity as was first thought, has turned out to be a soil-borne disease of considerable economic importance. The work of Cadman, Harrison and others has shown AMV to be the cause of several crop diseases, the most important being raspberry yellow dwarf, strawberry yellow crinkle, a disease of cherry trees, characterized by rasp-like excrescences on the leaves, and rhubarb mosaic. Harrison and Winslow describe a symptom not noted before in raspberry (Rubus idaeus L. var. Mailing Exploit) infected with AMV, this is that young infected
canes retain their leaves longer in autumn than healthy canes, so that in November infected areas appear as pools of green in fields of otherwise bare canes. Previously undescribed diseases in various plants were all found to be due to infection with AMV. These include a chlorotic blotching in white clover (Trifolium repens L.), stunting and mottling of celery and vegetable marrow and a bright yellow vein-banding on the leaves of wild elder (Sambucus nigra L.). In addition the virus has been obtained from runner bean (Phaseolus multi-florus Willd.), sweet clover (Melilotus officinatus Lam.), two common weeds, chickweed (Stellaria media Vill.) and annual nettle (Urtica wrens L.) and box (Buxus sempervirens L.). The nematode vector is another species of Xiphinema, X. diversicaudata (Micoletzky) and it has been shown to retain the virus for as long as twenty-four days in moist peat, free from plants. It is clear that the host range of both AMV and its vector is very wide and Harrison and Winslow suggest that both are common constituents of natural woodland in Britain and that their incidence has decreased since the advent of agriculture.

Another virus discovered some years ago as a casual infection of a tomato plant, and thought at the time to be of little significance, has turned out to be a soil-borne virus of some economic importance. A single blotched tomato fruit was sent by a grower to the writer at Cambridge and its possible virus content was examined by inoculation to differential hosts. No fewer than three distinct viruses were isolated by this means and it is somewhat surprising that a single ripe tomato fruit should contain three distinct and entirely different viruses, none of which is insect-borne. These were tobacco mosaic virus, potato virus X and a third virus, hitherto undecided. The
The separation of these three viruses affords a good illustration of the analysis of a complex of plant viruses by the use of differential hosts or filter plants as they are sometimes called. The first inoculation was made to plants of Nicottana glutinosa which, as we have seen in Chapter One, localizes the TMV on the inoculated leaf in the form of 'local lesions', potato virus X and the third virus moved out of the inoculated leaves and could be obtained free of TMV in the other parts of the plant. Inoculation was next made to a number of species, known to be immune to potato virus X, in the hope that they would be susceptible to the unknown virus. In the final result the French bean (Phaseolus vulgaris), nasturtium (Tropaeo-lurri) and the ridge cucumber all became infected without the presence of virus X. Sub-inoculation from these hosts back to the tomato plant produced a disease to which the name tomato black ring was given because of the development of numerous small black necrotic rings on the leaves.

Since this first occurrence tomato black ring virus and its related strains have been recorded from potatoes in Germany as potato bouquet, and as beet ringspot virus by Harrison from sugar beet, potato, turnip, oat, raspberry, strawberry and weed plants from Scotland, and, lately from lettuces in England. It has been shown by Harrison and his co-workers to be transmitted by the nematode Longidorus elongatus.

MITE-TRANSMITTED VIRUSES

We come now to another group of plant viruses with a distinctive type of vector which, although not an insect, belongs to the Arthropods. The first suggestion that mites were concerned with the spread of a plant virus was made
as long ago as 1927 and confirmed by Massee in 1952. This was the so-called reversion disease of black currants and it is transmitted by the mite Phytoptus ribis (Westw.), known as the 'big bud mite', a name derived from the swelling of the affected buds.

In the year 1955 several more examples of mite-transmitted viruses were recorded. Slykhuis demonstrated that the Eriophyid mite Aceria tulipae K. was the vector of a virus attacking wheat known as wheat streak mosaic. About the same time it was shown by Flock and Wallace that the virus of fig mosaic, a disease widespread in California, is transmitted by the mite A. ficus Cotte. Finally, the long-sought-for vector of the American peach mosaic virus has now been identified by Cochran and his colleagues as the mite, Eriophyes insidiosus Keifer.

Not much is known of the relationship between this type of vector and the viruses transmitted by them. Slykhuis has shown that when the mite Aceria tulipae was reared on wheat infected with streak mosaic all stages except the eggs carried the virus. However, it was found that when virus-free mites in different developmental stages were colonized on diseased wheat the nymphs could acquire the virus but the adults could not. This is a similar phenomenon to that already described earlier in this chapter where it was shown that the larval thrips can acquire the virus of tomato spotted wilt de novo, but the adult insect cannot.

**FUNGUS-BORNE VIRUSES**

We come now to a type of soil-borne virus of which the vector is an organism outside the animal kingdom altogether, in other words a root-infecting fungus.

The tobacco necrosis viruses form an interesting
group of soil-borne agents which infect the roots of many plant species without becoming systemic in their hosts. In the past it had been suggested that the vector might be a root fungus but the first circumstantial evidence of this was pointed out by Teakle in 1960 who showed that the fungus Olpidium brassicae was always closely associated with infection of plants by one of the tobacco necrosis virus group (TNV).

Further work by Teakle and his co-workers confirmed that the zoospores of this fungus did actually transmit the virus.

Moreover, they showed, by sterilization of the outside of the spore, that the virus was not merely a contamination of the spore coat but was contained within the spore itself which thus acts as a genuine vector of the virus.

A further point of interest lies in the fact that not all strains of Olpidium brassicae can transmit the virus; thus several crucifer strains have not transmitted TNV, but lettuce strains have consistently done so.
CHAPTER SIX

THE BACTERIAL VIRUSES

THE discovery of the bacterial viruses was the result of a chance occurrence, somewhat similar to the incident which led to the discovery of penicillin. This arose, it may be recalled, as a result of mould spores falling on an uncovered petri dish containing a bacterial culture. Alexander Fleming noticed that where the mould was developing on the agar, the bacteria were being lysed or destroyed. Instead of discarding the dish as useless, he investigated the matter further, and from this insight on the part of Fleming arose the tremendous use of antibiotics and all which that entails.

It was in 1915, some five years before Fleming's discovery, that Twort, an English bacteriologist was examining some bacterial cultures on a plate of agar or a similar medium when he noticed some clear areas developing in a culture of a Micrococcus, similar to those observed in 1910 by a French Canadian, d'Herelle, who called them 'taches vierges', literally translated 'virgin spots'. Twort, however, conceived the notion that here was a disease of the bacteria and proceeded to investigate the matter. First of all, he found that material transferred from the clear areas to fresh bacterial cultures induced the formation of more such areas or 'plaques' as they are called today; in other words he showed that the agent was infectious. Furthermore, he proved it to be filterable, showed that it multiplied, reproduced true to type, and that the number of plaques was inversely proportional to dilution. In other words, here was a virus which attacked
bacteria. Two years later this discovery was confirmed by d'Herelle who gave to the virus the name 'bacteriophage', meaning 'bacterium eater'; this term, in its shortened form of phage is still in use.

The bacterial viruses have since been most extensively studied, for the bacterium itself, being a single cell, is ideal for the investigation of the interrelationships between virus and host. Indeed, the subject of phage genetics is now a most important study with a voluminous literature of its own.

Bacterial viruses differ in some respects from other viruses; first they are apparently restricted to bacteria and do not infect any other type of organism. Secondly some of them contain a very high proportion of de-oxy ribosenucieic acid (DNA), nearly per cent., compared with the small proportion of DNA or RNA in other viruses. The third point concerns the shape of the virus particle which, in a large group of the phages, is tailed and of complicated construction. Until fairly recently it was thought that all bacterial viruses contained DNA and were of a tadpole shape with a 'head' and a 'tail'. Now it is known that there is great variation in shape and some phages are spherical or near spherical or even rod-shaped, resembling some of the insect or plant viruses. Both DNA and RNA types have now been described.

*Morphology*

Since, up to the present, most attention has been paid to the tailed phages we shall deal only with the morphology of this type. Much of our information is based on studies of what are called the T-even phages, especially T$_2$ and its bacterial host Escherichia coli.
The new method of negative 'staining' for the electron microscope, which is not really staining but consists rather in the enhancement of contrast, allows the ultrastructure or 'make-up' of the particle to be visualized. Brenner and Home and their colleagues in Cambridge developed a procedure for disjoining the even-numbered T-phages into their structural components, and this, together with the negative 'staining' with phosphotungstic acid, provides a wealth of structural detail, gives good contrast and preserves the three-dimensional morphology. Most of the following information is derived from their work.

The 'head' of this group of phages is in the form of a bi while the 'tail' is cylindrical and is the means of attachment of the phage to the bacterial cell. Recent work has shown the 'tail' itself to be a complex structure, and changes induced by various treatment, such as heat, freezing and thawing and hydrogen peroxide, include a shortening of the outer layer (the sheath) of the tail, revealing an inner core, coaxial with the sheath, which displays at its distal tip a hexagonal plate to which are attached the tail fibres. The effect of the hydrogen peroxide on the phage is to contract the sheath which becomes thicker and shorter than the uncontracted sheath. The core is attached to the hexagonal head which is filled with DNA (Fig. 4), but the sheaths appear unattached to the head in both normal and altered phages.

Electron micrographs of the contracted sheaths show them to be hollow cylinders, 350 Å long and 250 Å in diameter, the diameter of the inner hole being 120 Å. By comparison the diameter of the extended sheath is 165 Å and its length is probably equal to that of the core (800 Å). In electron micrographs of the purified contracted sheaths,
a number can frequently be seen standing on end, and these display a 'cogwheel arrangement of the sub-units whilst others lying obliquely reveal a helical grooving which suggests a helical arrangement of sub-units in the sheath. A number of striations have also been observed across the tail of the intact phage; there are about twenty-five of these striations in an extended sheath and their spacing is estimated to be 30-40 Å.

The cores are hollow cylinders 800 Å long and 70 Å in diameter, the diameter of the central hole being estimated to be 25 Å. The tail fibres are long thin structures, usually six in number, with a characteristic kink in the middle; they measure 1,300 Å by 20 Å.

By treatment of T₄ phages at a mild acid pH, followed by digestion with DNase (desoxyribonuclease) and trypsin, Brenner and his colleagues obtained a preparation of the empty 'heads' which retained their hexagonal shape; they were seen to consist of a thin membrane estimated to be about 35 Å thick.

The Infection Process

We have seen that the resting infective phage particle consists essentially of DNA surrounded by a coat of protein; the rest of the particle, the tail, tail fibres, core and sheath all being part of the rather elaborate mechanism employed in the process of infecting the bacterial host cell.

The phage particle attaches itself to the wall of the bacterial cell by means of the tail fibres and a specific receptor on the cell wall; after this initial reactor a more permanent bond of unknown nature is formed. The next step appears to be an alteration in the tip of the phage tail; the tail fibres unwind and are removed. It is thought that
this step is necessary for the exposure of the tail enzyme which hydrolyses the cell wall. We have seen previously that the tail sheath contracts and this is doubtless part of the mechanism for forcing the core through the bacterial wall. This core has been shown to be hollow, as we have seen, allowing the DNA in the head to pass down into the host. Very little is known about the mechanism responsible for the ejection of the DNA out of the head, but Kellenberger and his co-workers have shown that no DNA is released when phages are adsorbed on empty cells even when contraction occurs promptly.

Put very briefly the infection process of the T-even phages consists of adsorption to the bacterial cell wall by the phage tail, enzymic dissolution of the cell wall, contraction of the tail and injection of the DNA which, of course, is the carrier of genetic information. The empty protein 'head' is left outside, and is known as a 'doughnut' by American workers.

After infection of the bacterial cell by the phage there are two alternative developmental cycles, depending on the phage and the physiological condition of the host. It will be necessary here to introduce some specific terms and their meaning. With temperate phages, which can establish lysogeny, one of two alternative sequences of events follows; either there occurs an irreversible reaction that leads to synthesis of virus, proteins, maturation of phage particles, and lysis (breakdown) of the bacterium, or there is established the condition of lysogeny. In this state an immunity reaction sets in, which prevents the onset of lysis and the vegetative multiplication of phage, and permits the establishment of what is known as prophage in the bacterial nucleus. Such bacteria carrying the prophage are known as
lysogenic bacteria, and phage production by such bacteria is the result of the breakdown of immunity.

The other developmental cycle is that of the virulent or intemperate phage; this type redirects the biosynthesis in the infected bacteria entirely to the task of phage formation. The virulent phage cannot become lysogenic because it destroys the nucleus of the bacterial cell at an early stage of infection.

**Phage Development**

The following account of the growth of the T₂ phage particles is based upon the work of Edward Kellenberger; for a fuller description the reader is referred to Volume Eight of Advances in Virus Research, published by Academic Press, New York and London.

In the infected bacterial cell there are two fractions of DNA functionally distinct. One fraction, which forms the pool of vegetative phage, is a highly hydrated DNA-plasm while the other DNA is in a condensed form. The first visible effect of infection with the T₂ phage is the breakdown of the bacterial 'nuclei' or nucleoids. Simultaneously with the synthesis of phage DNA a new morphological feature develops in the bacterial cell; vacuoles of indefinite form, and filled with what appears to be DNA, can be found distributed in a haphazard manner throughout the bacterium. It is in these pools of DNA that phage-shaped bodies later begin to appear. Kellenberger calculates that the DNA concentration in the pool is only 1/15 of the DNA concentration in the phage heads; this explains the very different appearance of the DNA in the two places.

Observations by electron microscopy suggest the existence of a number of distinct precursor particles of phage. These seem to be first the condensed DNA, then
a fragile precursor phage without a tail, and the same with a 'growing' tail. (Fig. 5.)

The build-up or synthesis of the protein coat surrounding the phage particle has been studied by Koch and Hershey. They suggest that the membrane of the phage head is formed virtually complete from amino acids in about one minute, contains most of the protein intended for one phage particle and thus represents the principal phage-precursor protein structure. At the time of its formation the head membrane is filled with nucleic acid but is very unstable, easily breaking down. For a space of about five minutes the head structure persists in this unstable state inside the bacterium and then is completed during an interval of one minute to yield the stable, infective phage particle. It seems, therefore, that the completion of the phage is not only the addition of the tail parts but involves also changes in the head membrane.

A tentative scheme of the growth cycle of the T₂ phage as suggested by Kellenberger is shown below.

Tentative scheme of the growth cycle of T₂ phage after Kellenberger
(Advances in Virus Research, 8, 1961, Academic Press)
CHAPTER SEVEN

THE INSECT VIRUSES

The viruses attacking insects can be divided into two main groups, those which are occluded in protein crystals of different types causing the 'inclusion body' diseases, and those which are free in the tissues as in the virus diseases of plants and the higher animals. Fewer than half a dozen viruses of the second group are so far known, mainly because less attention has been paid to them, and because of the greater difficulty in diagnosis for which an electron microscope is necessary. The great majority of the insect viruses attack only the larval stages.

The inclusion-bodies are of two kinds, the polyhedra or many-sided crystals; diseases of this type are called polyhedroses, and the granules, the associated disease being a granulosis.

The polyhedroses are sub-divided into nuclear and cytoplasmic diseases, and we will discuss first the nuclear polyhedroses. The tissues of an insect infected with this type of virus are filled with many millions of polyhedral crystals. These polyhedra occur mainly in the skin, blood cells, fatbody and tracheae; as we shall see later this is important for differentiation between nuclear and cytoplasmic polyhedroses. The routine test for the presence of polyhedra is simple enough; a smear is made from the blood or tissues of the larva, fixed with mild heating over a bunsen flame and stained with Giemsa solution. If present, the polyhedra are easily visible under the oil-immersion
lens, unstained against the stained background. Polyhedra of this type have been known for many years, and were at one time the subject of much controversy over their nature, one opinion being that they were an organism of a new type and were the cause of the disease. However, two Czech workers, Komarek and Breindl, showed that the polyhedra would dissolve in weak alkali, and this liberated something just visible, under dark field illumination on the optical microscope, as bright dots vigorously vibrating with Brownian movement. They suggested that these dots and not the crystals were the true disease agent. This suggestion was confirmed in 1948 by Bergold in Germany who also dissolved the polyhedra with weak alkali and observed them in the electron microscope. He showed that the bright dots were actually bundles of virus rods. Here we may point out that all the viruses of the nuclear polyhedroses and granuloses, so far examined, are rod-shaped.

The viruses of the nuclear polyhedroses attack mainly the caterpillars of butterflies and moths (Lepidoptera), but several cases are also known in the larvae of sawflies (Hymenoptera) and one in a fly larva (Diptera). The symptoms of the disease are much the same in the various larval species. The developmental cycle of the disease is somewhat as follows. The polyhedra are ingested by a susceptible larva, usually through contamination of the food plant. Incidentally the polyhedra are an excellent means of transport for the virus since they are very resistant and comparatively unaffected by weather conditions. Moreover, they act as a protective covering for the virus itself which is rather labile and can be easily destroyed by an adverse environment.

On entering the alimentary canal of the larva the polyhedra dissolve in the alkaline juices of the gut and the
liberated virus enters the cells of susceptible tissues. The exact method of entry into the blood cells, for example, is not known. Once in the cell nucleus the virus begins to multiply and the polyhedra start to form; under pressure from the accumulating crystals the nucleus enlarges enormously and finally ruptures. By the time the nucleus ruptures it has already filled the cell, so that the latter also bursts and the polyhedra are liberated. As the disease progresses and the numbers of polyhedra continue to increase, the other tissues of the body break down and the whole of the body contents become liquefied. Finally, the skin which, by now, has become extremely thin and fragile, ruptures at a touch and the liquefied contents containing millions of polyhedra are liberated to contaminate the food plant and spread the virus far and wide. It usually takes ten to twelve days from the ingestion of the polyhedra for the disease to develop.

It is a characteristic of the inclusion-diseases of insects for the virus rods to be occluded in membranes and capsules. When the nuclear polyhedra are dissolved in weak alkali, it may frequently be observed that the virus rods are enclosed in an envelope containing bundles of five or more. The individual rods are also enclosed in a capsule, while the actual material of the virus is contained in a thin intimate membrane. As we shall see in a moment it is possible to break down the virus rods into some of their component parts so that the various membranes become visible.

There is much disagreement among the different workers as to how the rod-shaped viruses are replicated. The idea of a 'life-cycle' was first put forward by Ber-gold in 1950 who made an examination of virus suspensions under the electron microscope, and arranged a sequence of
all particles found into a developmental and multiplication cycle. Some years later in 1959 Bird suggested a somewhat similar developmental sequence.

These conceptions of Bergold and Bird are plainly influenced by ideas of the conventional development of organisms and seem out of step with the biosynthesis of other viruses.

By the use of dilute alkali the breakdown of the virus rods from the nuclear polyhedroses can be observed. The first stage in the liberation of the contents of the intimate membrane is the peeling off of the outer capsule. The capsules break in the centre and fold backward thus forming two spheres still joined in the middle. These finally break apart and are probably the same as Bergold's spherical sub-units which he said were discharged from the intimate membrane and formed part of the 'life-cycle'.

After the peeling away of the capsule the intimate membrane is exposed and it measures about 20 Å in thickness; it has a slightly different structure at either end. At very high magnification the contents of the intimate membrane give the appearance of a wide-spaced helix; these contents are discharged from either end of the intimate membrane and appear to uncoil as they flow out. This helix is thought to be, in part, nucleic acid (DNA). The actual assembly of the virus rods has not been observed, but the presence of a helical structure similar to that observed in the rod of tobacco mosaic virus strongly suggests a similar method of replication (see Chapter One).

Mention was made previously of there being one nuclear polyhedrosis recorded from the larva of a fly (Diptera). This is an interesting disease and it differs in several ways from the more usual nuclear polyhedroses
affecting the Lepidoptera. The larva attacked is the well-known 'leatherjacket' and the adult fly is the crane-fly or 'daddy-long-legs', Tipula paludosa Meigen. Only the blood cells are affected and in a late stage of the disease the larva appears to be packed with enormous numbers of blood cells. This may be a genuine increase in their number or may possibly be due to the fact that the blood cells become greatly enlarged owing to the presence in them of numbers of polyhedral crystals. These crystals have a very characteristic shape and are not, strictly speaking, polyhedra; they resemble more a half-moon or the segment of an orange. The behaviour of these crystals, when treated with weak alkali, is also very different from that of other polyhedra. Instead of readily dissolving and liberating the virus particles they elongate to several times their length; when the pH is restored to 7 they resume their normal shape. This process can be repeated indefinitely and takes place as rapidly as the solutions can be changed.

In such resistant polyhedra it is difficult to observe the virus particles within by attempting to dissolve away the crystal. So the problem had to be approached in a different manner; ultrathin sections of the polyhedra were cut and examined with the electron microscope. The sections revealed the presence of rod-shaped virus particles, each enclosed in an outer membrane; the particles appear similar to the rod-shaped viruses of other nuclear polyhedroses. There is, however, one point of difference; the particles inside the polyhedra seem to be arranged in regular rows instead of the haphazard agglomeration found in the nuclear polyhedra of other insect orders.

Turning now to the cytoplasmic polyhedroses we find a number of differences from the foregoing nuclear
diseases. The virus multiplies only in the cytoplasm of the gut cells, usually the epithelial cells of the midgut (Fig. 6), and from there spreads to the foregut and the hindgut. The skin is not attacked and the resulting disease is unlike that of the nuclear polyhedrosis. Infected larvae lag greatly behind normal larvae in their development and affected individuals can be recognized by their small size, loss of appetite, and, sometimes disproportionately large head and long bristles. Later, the polyhedra developing in the gut can frequently be observed through the dorsal integument as a pale yellow or whitish area. In late stages of the disease polyhedra are frequently regurgitated or voided in large quantities with the faeces, and this, of course, helps to spread the infection. On opening a caterpillar infected with a cytoplasmic polyhedrosis a characteristic sign is the opaque whitish appearance of the midgut compared with the green translucent gut of the healthy caterpillar.

We have seen that the routine test for the presence of nuclear polyhedra is to make a smear from the diseased larva on a microscope slide, fix with mild heating and stain with Giemsa's solution. The result is a stained background which shows up the unstained polyhedra. The reaction of the cytoplasmic polyhedra is different since they readily take up the stain. It is important, however, in making a smear from a larva infected with a cytoplasmic polyhedrosis, to make sure that the midgut is punctured otherwise no polyhedra will be liberated.

Furthermore, the polyhedra differ sharply in their reaction to dilute alkali; instead of dissolving completely and leaving behind rod-shaped virus particles enclosed in a transparent membrane the cytoplasmic polyhedra dissolve only partially and a sponge-like
residue remains honeycombed with the sockets in which the virus particles have rested. There is no encircling membrane. The shape of the empty sockets, which are near-spherical instead of being rod-shaped as in the nuclear polyhedra, gave the first indication that the virus of a cytoplasmic polyhedrosis was also near-spherical in shape.

The isolation of the virus itself from cytoplasmic polyhedra is more difficult than with the nuclear type because of the greater resistance of the former to the action of weak alkali. The time allowed for the polyhedra to dissolve in sodium carbonate is very critical and is only a matter of seconds; critical too is the concentration of alkali. After the polyhedra are dissolved the bulk of the fluid is greatly increased and the solution is then subjected to several cycles of centrifugation until a pellet of pure virus is obtained.

We have seen previously in dealing with the small plant viruses that the particles are not spherical in shape but icosahedral having twenty sides, and the same is true of the viruses of the cytoplasmic polyhedroses. Electron micrographs of a particle which is an icosahedron will show a silhouette of five or six sides, and this is more clearly demonstrated in the case of the Tipula iridescent virus which is discussed a little later. Some pictures, however, of virus particles from a cytoplasmic polyhedrosis affecting a large silkmoth larva, Antheraea mylitta Drury, show not only the six-sided contour of the particle but also a similar-shaped socket in the polyhedral crystal from which the virus has come. The polyhedral shape of the virus sockets in the crystal has been further demonstrated by means of carbon replicas of the polyhedra after removal of the virus particles.
An ingenious method of demonstrating the contour of a virus particle is by a technique of electron microscopy known as double shadowing or shadow analysis. This is explained in the description of the Tipula iridescent virus but its application has shown that the cytoplasmic virus of A. mylitta is in fact an icosahedron.

As regards the structure of the cytoplasmic polyhedrosis viruses this seems to be very similar in the half dozen or so examined by the writer and his colleagues and lends colour to the possibility that most of these viruses are one and the same. The virus particle has an icosahedral core of twelve knobs surrounded by a membrane; this is unstable and at an alkaline pH breaks into two halves consisting each of one knob surrounded by five others attached to half the membrane. In all the types studied the particles have a diameter of 600-650 Å and are associated with polyhedra of the shape of rhombododecahedra.

The second type of inclusion-body disease, the granulosis, has only been known for a comparatively short time, being first recorded by Paillot in 1926. He found it infecting the larvae of Pieris brassicae L., and named it 'pseudo-grasserie'. The first study of the disease by modern methods, using the electron microscope, was made in the U.S.A. by Steinhaus in 1947, and he suggested the name of granulosis for the disease. The name is derived from the presence in the tissues of the infected caterpillars of millions of tiny granules which are just at the limit of resolution of the optical microscope using a 1/12 in. oil immersion lens. Actually each of these granules is a minute crystal and it contains usually only one rod-shaped particle and occasionally two. This is in marked contrast to the polyhedra each of which contains many hundreds of virus
particles. So far the granuloses have not been recorded from any insect order except the butterflies and moths, Lepidoptera; the virus only seems to attack the larval stages.

The symptoms of the granuloses resemble closely those of the nuclear polyhedroses, largely because the skin is attacked in both cases. The body contents become liquefied similarly and in the last stages of the disease the larvae hang by their abdominal feet, frequently in an inverted V; the skin ruptures at a touch and the granules are liberated in enormous numbers to contaminate the food plant and thus spread the disease.

In the granuloses the fat body seems to be the main focus of virus development, although, as the disease progresses, the blood cells, skin and tracheae are also affected. There seems to be some doubt as to whether the virus multiplies in the nucleus or the cytoplasm or both, although granules seem to be present in the nuclei of the fat cells in the granuloses of Pieris brassicae, the large white butterfly and Melanchra persicariae, a 'cutworm'. According to some German workers formation of the granules takes place in both nucleus and cytoplasm.

When the granules are treated with weak sodium carbonate and viewed in the electron microscope they are seen to have collapsed into a thin 'ghost' revealing their contents which consist of a rod-shaped capsule. Further treatment with alkali shows the virus rod surrounded by its intimate membrane inside this capsule.

The morphological detail of the virus rod extracted from the granulosis disease differs in a slight respect from the virus rod of the nuclear polyhedrosis. The intimate membrane shows a close-packed helical structure but whether this is formed by the membrane or its contents is not yet clear. However, this helix has not been resolved on
the intimate membrane when emptied of its viral contents. Another point of difference from the virus rod of the nuclear polyhedrosis is the presence of a head capsule at either end of the intimate membrane, the purpose of which is not known.

The last group of insect viruses consists of the free viruses, so called because they lack the inclusion bodies and capsules and lie freely in the cells of the infected insects. As mentioned already, not many of this type of virus have been described but there is no reason to suppose that many more will not be discovered in the future. From a species of armyworm or processional caterpillar, Pseudaltea unipunctata Haworth, a small near-spherical virus measuring approximately 25 mµ has been isolated. The caterpillars infected in the late third instar appear swollen and somewhat darker than normal. The cuticle of the diseased larvae has a waxy appearance, and in some cases the middle part of the insect is slightly enlarged. There is none of the disintegration and breakdown characteristic of the nuclear polyhedroses.

In the Hymenoptera the honey bee, Apis mellifera L., is known to be susceptible to two non-inclusion body viruses. One which appears to be a very small spherical virus attacks the adult bee causing paralysis; the second causes a disease in the larva known as 'sacbrood'. Very little is known of this virus and it has not been isolated.

A non-inclusion disease of which the histology has been studied is the so-called 'Wassersucht' of cockchafer grubs Melolontha spp. This is similar to a previously described condition known as 'Heidenreich's disease' or 'histolytic disease' of Oryctes spp. In the fat body of infected larvae, the albuminoid spheres normally present are apparently transformed into virogenetic stroma and give
rise to virus particles which appear to be spherical and contain ribonucleic acid (RNA).

Two non-inclusion virus diseases, and a possible third, have been described in arthropods outside the Insecta. These occur in the spider mites (Arachnida); in the citrus red mite, Panonychus citri McGregor, and in the European red mite, Panonychus ulmi Koch.

In the citrus red mite the virus appears to affect the nervous system, since diseased mites become paralysed with the legs held in a stilt-like manner. A curious feature of the disease is the almost invariable appearance, in the body of the infected mite, of numerous birefringent crystals which are not virus crystals but appear to be the result of a disordered metabolism.

A non-inclusion virus of great scientific interest was discovered at Cambridge during routine examination of large numbers of larvae of the fly Tipula paludosa Meigen for the presence of another virus, that of the nuclear polyhedrosis already briefly described.

A larva affected with this virus can be recognized at once by the change in appearance that it induces in the body of the affected host. Whereas the normal appearance of the larvae of T. paludosa is a dark tan, the colour of the diseased larvae is a somewhat opalescent blue-indigo. Examination by low-power microscopy shows that the colour is particularly intense in the lobes of the hypertrophied fat body; as the disease approaches its termination the colour is less intense and is more generally dispersed throughout the body. On opening a larva in an advanced stage of the disease, a thin white fluid is liberated consisting of the disintegrated fat body together with a highly concentrated suspension of the virus which can be recognized immediately by its brilliant iridescent colours.
From these peculiar optical properties the name Tipula iridescent virus (TIV) is derived.

Thin sections of fat body from a larva infected with TIV have an unmistakable appearance when examined with the electron microscope. The cell cytoplasm is completely filled with virus particles which, in an advanced stage of the disease, will already be orientated into a crystalline array. It is these microcrystals formed in the living insect which produce the brilliant iridescent colours.

Although the fat body is the initial site of multiplication of the virus, as the disease progresses other organs become infected. Indeed, the virus seems able to multiply in many types of insect tissue including the muscles, wing-buds, legs and head.

For a number of years the opinion has been held by some workers that insect viruses were strongly species-specific and that it was not possible to pass a virus from one species of insect to another. Experiments along these lines were liable to have inconclusive results because of the possibility or even likelihood of the appearance of a second virus from a latent infection already present in the inoculated insects. Such latent infections are liable to be stimulated into virulence by the addition of a second virus. Under these circumstances it is difficult to differentiate between a genuine cross-transmission and the stimulation of a latent virus infection. This difficulty, of course, is partly due to a lack of a ready means of recognition of the different viruses involved.

What was needed to solve the problem of whether insect viruses were cross-transmissible between species was a virus which had such definite characteristics that it could not be mistaken for any other virus. TIV is a virus of this type, and infection by it can be immediately recognized
by the blue or violet iridescence of the affected organ, by the characteristic pentagonal or hexagonal outline of the virus particles under the electron microscope, and by the enormous quantities of virus produced in the diseased insect.

In a series of experimental infections TIV has now been transmitted not only to half a dozen different species of Diptera, but outside the order altogether to about twelve species of Lepidoptera and three of Coleoptera.

The TIV particle is rather large and extremely uniform; it lends itself well to the technique of shadow analysis or double shadowing whereby the actual topography of the particle can be demonstrated.

When virus particles are examined in the electron microscope they are liable to be flattened owing to the forces of surface tension; in order to avoid this the technique of freeze-drying was developed. Frozen-dried particles retain their natural shape and this is essential for the double-shadowing process.

A particular type of polyhedron, if it is regular, should cast a particular type of shadow, if it is cast with a specified orientation with respect to the positions of the vertices of the polyhedral particle.

Williams and Wyckoff developed a method of shadow-casting in which metal, evaporated from an electrically heated filament in a vacuum, impinges upon a specimen from a sufficient distance so that the metal atoms arrive in almost parallel straight lines. The metal, being more opaque to the electron beam casts a shadow, thereby giving a three-dimensional effect to the object.

In order to prove that the TIV particle is actually an icosahedron, the double-shadowing was carried out as follows. A model of an icosahedron was made and
shadowed by two light sources separated 60° in azimuth and orientated so that an apex of the hexagonal contour points directly to each light source. This throws two shadows, one is four-sided and pointed and the other is five-sided with a blunt end. A particle of TIV frozen-dried and shadowed in the same way is shown in Fig. 11(b); the similarity between the shadows thrown is evident. This indicates with fair certainty that the TIV particle is an icosahedron.

The purification of TIV is very simple chiefly owing to the large size of the particle and the absence of particles of comparable size in the extracts of diseased larval tissue. The large amount of virus in the insect, 25 per cent. of the dry weight, also helps. The actual procedure of purification consists mainly of preliminary clarification of the extract by low speed centrifugation, followed by two cycles of high- and low-speed centrifugation. The pellets of virus resulting from centrifugation have fascinating optical properties. By transmitted light the pellet appears an orange or amber colour. By reflected light it has an iridescent, turquoise appearance. Within the pellet may be seen small regions reflecting the incident light quite brilliantly, giving the entire pellet the appearance of an opal.

Although a cytoplasmic virus, TIV has been shown to contain about 13 per cent. of deoxyribosenucleic acid (DNA).

High-resolution electron micrographs of thin sections of the TIV particle have suggested that there was a double membrane enclosing an inner core. More recent work, however, using the negative staining technique with phosphotungstic acid, PTA, shows that the apparent second membrane is actually an inner row of protein sub-units.
As with a number of other viruses (see Chapter Two) numerous empty particles occur and these have been studied with the electron microscope using the negative PTA staining to try to elucidate the structure of the protein coat of the TIV particle. This seems to be composed of 812 protein sub-units with which are lipids apparently helping to bind the sub-units into a rigid structure. The sub-units measure 85 Å by 140Å and are hollow and hexagonal when viewed end-on. They are arranged to form a twenty-sided solid figure (ico-sahedron), as we have already seen, each side being an equilateral triangle.

The central core is also an icosahedron composed of forty-two large knobs which is surrounded by the protein coat or membrane mentioned above. The location of the DNA in the TIV particle has not been exactly identified, but sections of the complete particle suggest that it is sandwiched between the outer membrane and the core (Fig. 7). On the other hand, sections of incomplete particles in tissue fixed at an early stage of infection suggest that there is a DNA-rich material forming a central structure of twelve sub-units.
PART TWO

Viruses affecting Man and the Higher Animals

CHAPTER EIGHT

THE PICORNAVIRUSES (ENTEROVIRUSES)

In the preceding chapters we have dealt with the viruses attacking plants, bacteria and insects. In the remainder of the book we are concerned with the viruses which affect man and the higher animals.

One of the pressing needs of virology as a whole is for a stable system of classification which would bring some kind of order into the vast collection of viruses so far recorded. There have been several attempts to erect such a system, one in particular utilized the Linnean binomial system of Orders, genera and species. However, these courageous attempts have broken down, largely owing to the lack of sufficient knowledge concerning the basic characteristics of the viruses themselves, which alone offer a firm foundation for a classification. It is fairly obvious that the properties of the virus itself are the main criteria and not the diseases caused which present too many variable factors, although these are also useful as a subsidiary aid.

Nevertheless some degree of order among viruses affecting the higher animals has been achieved by
arranging them into groups according to certain specific properties. Thus, Andrewes quotes the following fundamental characters, nucleic acid (whether DNA or RNA), size in millimicrons, number of capsomeres (the protein sub-units constituting the outer coat of the virus particle), presence or otherwise of a membrane surrounding the capsid (the whole virus particle), site of multiplication (whether in nucleus or cytoplasm), maturation at cell surface, an interesting feature of the myxoviruses, and ether sensitivity.

Using these criteria, the viruses attacking vertebrate animals have been arranged into a number of groups, the Pox viruses, Herpesviruses, Myxoviruses, Arboviruses (arthropod-borne) and Picornaviruses which used to be known as the enteroviruses. The word Picorna is derived from 'Pico', meaning 'very small' and RNA indicating the type of nucleic acid composition. In his last group is included the important and well-known virus of poliomyelitis usually called poliovirus, which is dealt with in this chapter. For an up-to-date account of the classification of the viruses of vertebrates, the reader should consult C. H. Andrewes (1962), Advances in Virus Research, 9, p. 271 published by Academic Press and also a book by the same author Viruses of Vertebrates published by Bailliere, Tindall and Cox, 1964.

We mention later the work of Enders and his colleagues who were the first to grow the poliovirus in non-nervous mammalian cells in vitro and how this work led to the production of a vaccine. It may be helpful if we give here a brief description of the modern method of growing animal viruses in tissue culture; much of this information is taken from a recent review on the use of tissue culture in virus research by Ross and Syverton (Ann. Rev. Microbiol., 11, 459, 1957). An important development was the
application of trypsin digestion to tissues whereby the cells became dispersed in a suspension in the place of fragments of tissue. The cells are cultured in a variety of growth media such as yeast extract for HeLa cells, lactalbumin hydrolysate for monkey kidney and other cells, and for the cultivation of human cells, horse or calf serum, or bovine allantoic and amniotic fluids are used. Yeast extract, lactalbumin hydrolysate and calf serum may all be incorporated in the same medium. The culture of plant and insect cells has not reached the high level attained in the tissue culture of the cells of vertebrate animals, but some progress is now being made, particularly in the case of insect tissues.

There are three main branches in the technique of tissue culture, the development of 'lines' or strains of cells in continuous culture, the maintenance of stock cell cultures and the mass preparation of cell cultures for the production of virus in quantity for purification, electron microscopy and so forth, and also for the quantitative study or assay of virus. The cultivation of animal cells is employed in two main forms, as sheets of contiguous cells about one cell thick, known as 'mono-layers', and suspensions of dispersed cells in a fluid medium.

There have been various developments in the tissue culture techniques for special purposes and an important one is that of Dulbecco and Vogt who initiated the quantitative assay of animal viruses by plaque count; this is somewhat similar to the method of counting the plaques or clear areas in sheets of bacterial cells made by the phages. Dulbecco and Vogt localized the spread of the destructive effect of the virus by overlaying the cell sheet with agar. By this means they were able to titrate the poliovirus in kidney cells, each plaque or focal lesion being
caused by one virus particle thus giving the assay a considerable degree of accuracy. Another important development in technique was made by Lwoff and his co-workers who adapted a micromanipulator to the study of virus propagation by individual isolated cells, and were able to relate stages of virus release to cytopathology. It was the work of Enders and his colleagues which first showed the cytopathic effects (CPE) of viruses on cells and that the growth of the virus could be recognized by its CPE. The behaviour in tissue culture is an important property for the characterization of new viruses and for their classification into related groups. The most important cytopathic effects are those which are based on more fundamental characteristics of the virus such as the site of multiplication, the method of growth of the virus and the way it is liberated from the cell. These processes can usually be recognized from the manner of the cell degeneration or by the formation of 'inclusion bodies' which are highly characteristic of the action of certain groups of viruses.

In the case of poliovirus there do not appear to be any very specific cytopathic changes in the cell and they consist primarily of cytoplasmic breakdown and nuclear lobulation. The virus is located exclusively in the cytoplasm and may occur as large crystalline aggregates of spherical particles.

It has been mentioned earlier that the myxoviruses are assembled at the cell surface and released continuously over a period of several hours. With polio-virus the release from the cell is different. Lwoff and his colleagues, who studied virus release from individual cells, have shown that the main bulk of the virus is released within a period of about an hour by what has been called a 'burst-like' process.
The whole subject has recently been discussed in two review articles, the cytopathic effect of animal viruses by H. G. Pereira and the plaque assay of animal viruses by P. D. Cooper. Both these articles are in Advances in Virus Research, 8, 1961, Academic Press, New York and London.

Although dating from pre-Christian times, poliovirus has only appeared in epidemic form in the last seventy-five years.

The disease it causes is called 'acute anterior poliomyelitis' because of the tendency of the virus to attack the anterior part of the grey matter of the spinal cord. The popular name of 'infantile paralysis' arose from the fact that the virus originally attacked mainly infants but in modern times young adults are equally susceptible. That a virus was the causal agent of poliomyelitis was first shown in 1909 by Landsteiner and Popper who reproduced the disease in rhesus monkeys by intracerebral injection with a bacteria-free filtrate from the spinal cord of fatal human cases.

The serious nature of the epidemics of poliomyelitis during the past two decades and the urgent need for more research on the causal virus resulted in the accumulation from voluntary contributions of huge sums of money for this purpose, particularly in the U.S.A.

From the great amount of research thus sponsored our knowledge of both the disease and the virus has been much increased. From the practical point of view the most important result was the cultivation of the virus in non-nervous mammalian cells in vitro, by Enders and his colleagues in 1949. Out of this tissue culture work arose the production of a vaccine against the "disease in man.

Besides the production of a vaccine this development in the field of tissue culture made possible a
better means of purifying the virus itself than the old method of extracting it from the central nervous system of a diseased host. This enabled the virus particle to be isolated and characterized on the electron microscope.

The method for the purification of poliovirus from tissue culture fluid, briefly given below, is taken from the work of Schaffer and Schwerdt. The virus was first precipitated out of the tissue culture fluid by the addition of 15 per cent. methanol; it was then sedimented by several cycles of ultracentrifugation. Finally the virus thus concentrated was fractionated by sedimentation in the ultracentrifuge through a sucrose density gradient (see page 118). This yielded several zones or bands in the centrifuge tube and infectivity was finally associated with the lowest band which contained a homogeneous population of spherical particles.

We have seen in the chapters dealing with the plant viruses how purification of several resulted in their crystallization, and this has been known for a number of years. The fact of crystallization was sometimes put forward as a fundamental difference between the plant and animal viruses, since, until recently, all attempts to crystallize the latter had failed. In 1955, however, Schaffer and Schwerdt succeeded in obtaining polio-virus in crystalline form; since then one or two other animal viruses, for example the Tipula iridescent virus (see Chapter Seven), have now been crystallized. In this connexion Klug and Caspar point out that from the X-ray results, it is clear that there are no structural grounds for distinguishing the smaller animal viruses from the plant viruses. - Crystallization of the MEF-1 strain of poliovirus
was accomplished by collecting a pellet of highly purified virus by ultracentrifugation and gently rocking the pellet with unbuffered saline at pH 5.9 overnight in the cold.

Steere and Schaffer, using the Mahoney strain of poliovirus, succeeded in preparing better crystals by crystallizing directly from a highly purified and concentrated suspension rather than from a pellet. Observation and measurement of the facial angles showed the crystal habit to be an octahedron with cube faces. The crystals were determined as of the isometric system (three axes of equal length at 90° to each other). Isometric crystals would be expected from particles of spherical or highly symmetrical shape.

By means of the frozen replica technique of Steere an electron micrograph was obtained of a factured, frozen crystal of Type 1 (Mahoney) poliovirus. In this micrograph the major planes seen are in closely packed square arrays; between the square-packed planes are sloping planes of hexagonal array. In the isometric system such arrays are found only in face-centred cubic packing; this type of arrangement, where each particle has twelve nearest neighbours, is the closest possible three-dimensional packing for spherical objects. Some of the plant viruses such as those of tobacco ringspot, squash mosaic and southern bean mosaic, when crystallized, also show face-centred packing. On the other hand, crystals of turnip yellow mosaic virus have been shown in X-ray diffraction studies by Klug and his co-workers to be body-centred cubic (see p. 65).

Chemical analysis has shown the nucleic acid of polio-virus to be ribonucleic acid, which appears to represent about 30 per cent. of the particle. Further studies on the RNA are dealt with later in this chapter.
The new negative staining technique for electron microscopy using potassium phosphotungstic acid, developed by Brenner and Home, has greatly enlarged our knowledge of the structure of virus particles. In a recent paper Home and Nagington have employed this method in a study of the poliovirus particle and its mode of development.

Three classical strains of the virus were grown in tissue culture using monolayers of HeLa cells (a continuous line of cells derived originally in 1951 from a cancer of the cervix uteri), and preliminary studies have revealed the presence of three kinds of virus particle, complete, incomplete and empty shells. Examination of infected cell fragments under the electron microscope showed that no particles, which had a definite resemblance to poliovirus in shape and size, could be distinguished until about 4-4 1/2 hr. after infection. At 3-4 hr. however, a number of large bodies could be seen measuring about 1µ across; no virus particles could be distinguished inside these bodies but their internal structure consisted of small particles of the same size as the sub-units observed in the actual virus particles. Later, recognizable virus particles could be detected inside these well-defined bodies which were bounded by a membrane; these bodies vary in size and contain from 10 to 500 particles in different stages of development.

From approximately 5-5 1/2 hr. after infection of the HeLa cells the three kinds of particle, mentioned above, can readily be distinguished. These are the incomplete particles which appear to have a partially assembled shell of sub-units, empty shells or 'ghost' particles and complete or nearly complete particles. It is considered probable that these three kinds of particle correspond to the zones or
bands which, as previously mentioned, were observed by Schwerdt and Schaffer in their sucrose gradient ultracentrifugation tubes.

The empty shells are thought to be comparable with the top component of turnip yellow mosaic virus (TYMV), see Chapter Two. After 6 1/2 hr. the large bodies containing the virus particles are seldom seen and it is thought that after this time they may break down. This fits in with the migration of the virus particles across the cytoplasm and their appearance in the extracellular fluid at about 7 1/2 hr.

It is interesting to compare the particle of poliovirus with that of turnip yellow mosaic virus since they appear to be very similar in their external structure. Klug and his co-workers have examined both viruses by X-ray diffraction methods. They consider that the protein shell is similar in each case, possessing icosahedral symmetry (532). The electron micrographs taken at high magnification by Home and Nagington show individual poliovirus particles, apparently in various stages of assembly and the sub-units can be clearly seen. Measurements of the particles seen inside the large bodies previously mentioned, and of particles free in the cytoplasm show them both to be 300 A (= 30 mµ) in diameter, a value comparing well with the dimensions predicted from the X-ray diffraction studies.

It was found difficult to resolve exactly how the protein sub-units in the poliovirus particle are arranged probably because they are so closely packed. However, in some cases, it was possible to make out five sub-units grouped around a hollow centre, each sub-unit being estimated at about 50 A. (5 mµ) in diameter.

In Chapter One we learned that workers in Germany and the U.S.A. were able to prove that the ribonucleic
acid (RNA) of tobacco mosaic virus, TMV, after separation from its protein coat was by itself infectious. When inoculated into a susceptible plant it was able to replicate and give rise to the normal mosaic disease accompanied by the complete virus particles. As may be imagined this discovery spurred on virologists to see if the same phenomenon was true also with other RNA viruses. In 1959 three Belgian workers (de Somer, Prinzie and Schonne, Nature, 184, 652) isolated infectious RNA from poliovirus by extraction with water-saturated phenol", the method used by Gierer and Schramm in Germany to isolate the RNA from tobacco mosaic virus. There has been slight evidence that, when bereft of its protein coat, the RNA of tobacco mosaic virus was able to infect plants which were not susceptible to attack by whole TMV. With perhaps this idea in mind the Belgian workers investigated the possibility of adapting poliovirus type I to the chick by inoculating infectious RNA into embryonated eggs. They hoped that by losing its protein coat, and with it numerous specific properties linked to the protein component, the infectious RNA would also lose its specificity for certain cells, and thus be able to invade cells insusceptible to poliovirus. They also hoped that the progeny of this infectious RNA might consist of a strain of virus with different virus-cell relationships which could be adapted to another host.

The experiments were successful in that the poliovirus RNA was proved to be infectious and to have multiplied in the embryonated eggs and to have given rise to complete virus particles. They were not successful in that the poliovirus present in the allantoic fluid was not infectious for the chick so that the original aim of adapting poliovirus to the chick by this method did not materialize.
With many viruses the terms 'infection' and 'disease' are by no means synonymous. This is due to the phenomenon of latent virus infection which is discussed more fully in Chapter Ten dealing with herpesvirus. Latent virus infection may be of more than one kind and with the poliovirus it is known as sub-clinical or inapparent infection. Here the poliovirus enters the intestinal wall and multiplies there but progresses no further. The serious symptoms of disease from which the name of infantile paralysis was derived are caused by the damage done when the virus enters the motor nerve cells of the anterior horn of the spinal cord.

Before concluding this brief description of the poliovirus it will be appropriate to give a very short account of the success recently obtained with a vaccine against the dreaded disease of poliomyelitis. Vaccines in use against virus diseases are of three kinds, first a similar or related virus which produces only a mild reaction but nevertheless stimulates the production of antibodies which give a solid immunity against the more virulent virus. The classical example of this type of reaction is inoculation with vaccinia virus against smallpox, from which the word 'vaccination' is derived.

The second type of vaccine consists of an altered or attenuated strain of the actual virus against which protection is needed. The routine protection of millions of people against the mosquito-borne virus causing yellow fever is achieved by inoculation with an attenuated strain of the yellow fever virus. This attenuated virus, known as 17D, has been propagated in chick embryo tissue and is a good example of a 'live' vaccine.

The third type of vaccine is made from an inactivated or 'dead' virus, and this has been used extensively in recent years"to combat poliomyelitis. Known as the 'Salk Vaccine',
after Jonas Salk who was responsible for its production, it is produced from poliovirus grown in tissue culture and afterwards inactivated by treatment with formaldehyde. This conversion of the virus to a non-infectious form is effected in 1:4,000 dilution of formalin at pH 7 and at 37°C. in about a week. It is necessary, of course, for the inactivation process to be carefully carried out in order to be certain that, on the one hand, there is no active virus left and, on the other hand, that the inactivation is not carried so far that the antigenicity (the power to stimulate antibody production) is destroyed.

The disadvantage of this type of vaccine is the large quantities needed since it does not of course multiply in the body; this is in contrast to the other two types of vaccines in which the virus does multiply and stimulates the production of more antibodies. In these cases a very small dose is needed as in vaccination against smallpox where a scratch made through a drop of fluid on the skin is sufficient.

Nevertheless, the Salk vaccine protects to a great extent against the disease and many millions of children and young adults in several countries have received the vaccine.

There has been a good deal of controversy as to the respective merits of a 'live' or 'killed' vaccine against poliomyelitis. The prerequisites for a 'live'-virus vaccine are the production of an attenuated strain and knowledge of the degree of the stability of the strain after inoculation to the human body. It is clear that the stability is of paramount importance since a reversion to virulence would be disastrous not only to the inoculated person but to his contacts and the neighbouring population. It is relevant to
mention here that in Canada the Dominion Council of Health has recommended that the Salk vaccine 'remain, for the present, the basis vaccine for protection against poliomyelitis for all age groups'. However, at the time of writing, large-scale immunization has been undertaken in Pennsylvania, U.S.A. by the use of the Sabin attenuated 'live'-virus vaccine taken orally and not by injection.
CHAPTER NINE

THE POX VIRUSES (VACCINIA VIRUS)

BEFORE we consider the virus in greater detail it will be well to review briefly its historical associations. The name of Jenner, a country doctor, will always be re-membered as that of the pioneer who in 1796 used cowpox virus or vaccinia to immunize against smallpox. There was a common belief among country people that the women who milked the cows and had suffered from cowpox never became infected with smallpox. To test this idea he inoculated a boy with the cowpox virus and then later followed that up with a dose of smallpox virus; the boy remained healthy. Vaccination, which gets its name from vaccinia virus, is now a general term and is not confined to immunization against smallpox.

Downie (Viral and Rickettsial Diseases of Man, eds. Rivers and Horsfall) considers that vaccinia is a virus of uncertain origin; it is now propagated in laboratories and is used mainly for prophylactic vaccination against smallpox. While certain strains may have originated from smallpox virus it is probable that most of the strains now used have originated from the virus of cowpox.

Vaccinia virus has a wide host range; apart from man, calves, sheep and rabbits have been regularly used in the preparation of virus in the production of vaccine lymph for smallpox vaccination. Other laboratory animals such as monkeys, rats, mice, guinea pigs and hamsters may also be infected but are less susceptible than the rabbit. Successful
attempts to grow vaccinia in tissue culture were made as long ago as 1925 by Parker and Nye who used rabbit kidney tissue; they showed that there was a fifty thousand-fold increase in litre of the virus in the culture fluid; this was before the chorio-allantoic membrane of the developing chick embryo had been used for the growth of viruses.

The pox viruses, of which vaccinia is representative, all develop in the cell cytoplasm, they resemble each other morphologically and appear to undergo a similar type of developmental cycle in the cell. In the hydrated state they appear to be ovoid and are brick-shaped when shadowed for electron microscopy. They are mostly large viruses measuring 250-330 m in breadth.

So far as is known all the pox viruses contain DNA which is probably situated within the 'core' or nucleoid of the particle; the structure of the vaccinia virus particle will be dealt with in more detail later. Vaccinia has been chosen as an example of a very large virus, so large indeed that it comes just within the resolution of the optical microscope.

Andrewes (Advances in Virus Research, 9, 1962) classifies the pox viruses into several sub-groups:

1. Variola-like viruses
   - Variola
   - Vaccinia
   - Monkey pox
   - Ectromelia
   - Cow pox

2. Other poxes affecting ungulates
   - Contagious pustular dermatitis (orf virus)
   - Goat pox
   - Sheep pox
   - Horse pox
   - Swine pox
   - Camel pox
   - Papular stomatitis (bovine)
Andrewes points out that the viruses of sub-group I, those related to variola, are very similar as judged by various immunological tests. Sub-group 2 viruses are less closely related to variola, and the avian pox viruses are immunologically quite distinct from the mammalian ones.

For purifying vaccinia virus the rabbit is generally used although the infected chorio allantois may also serve for the preparation of virus suspensions, but in this case there is some difficulty in freeing the virus particles from the tissue.

A concentrated suspension of a rabbit-adapted strain of vaccinia virus is rubbed into the skin of a rabbit, and this is harvested three or four days later. By this procedure a large quantity of highly infectious starting material can be obtained. Further purification procedures consist of alternate cycles of high- and low-speed centrifugation.

Vaccinia has a complex chemical structure. Protein, nucleic acid (DNA), phospholipid, neutral fat and carbohydrate have been found in relative amounts similar to those of bacteria and mammalian cells.

Owing to its large size, about 250 mµ, the vaccinia virus particle is easily visualized on the electron
microscope and the early shadowed electron micrographs showed it to be brick-like in shape, with a dense spherical central region. The studies of Nagington and Home by means of thin sections and with the negative staining technique, using phosphotungstic acid, have greatly increased our knowledge of the fine structure of the virus, which seems to have features not observed in other viruses. In view of the large size of the vaccinia particle a more complicated structure would be expected. Nagington and Home suggest that there are two main types of mature particles: the first is electron-dense, but the shape varies from roughly rectangular to loaf- or thimble-shaped. It is normally symmetrical about the minor axis only and is 303 mµ, long by 240.3 mµ, wide. There is frequently a round central opaque region about 100-150 mµ, across and a crescentic opaque area on each side; between them there appears to be some penetration of PTA, possibly as a result of a more permeable structure. These particles generally show clearly defined thread-like structures, usually nearly at right angles to the particle margin and often they can be seen curving round it. They are 60-80 A in diameter; a less dense central region makes them appear tubular. The second major type of particle is more regularly rectangular than the first and is larger by about 50 m, in length and breadth. It has a well-defined outer region about 250 A wide which probably accounts for the size difference. In this can sometimes be seen a radial pattern of projections about 30 A wide and about 30 A apart. No internal structure could be seen. Noyes has made a parallel study on the structure of vaccinia virus, and he suggests that the different forms described by Nagington and Home are all mature particles but with the surface structure increasingly disorganized by the purification methods and by increased
penetration by PTA. In particles of the virus ruptured by sonification (high-frequency sound waves) fine filaments, 20-25Å in diameter, were observed which Noyes considered to be part of the nucleoid and might be DNA.

Nagington and Home made some studies on the development of the vaccinia virus particle; they find that the earliest forms appear to be empty, and maturation is accompanied by the accumulation of electron dense material within them. This occurrence of empty shells or 'ghosts', particularly in early stages of disease, is characteristic of certain viruses, notably turnip yellow mosaic virus, poliovirus and the insect virus known as TIV (Tipula iridescent virus). The interest lies in the significance of these empty particles. Are they incomplete in the sense of being misfits or unsuccessful attempts at particle formation or are they steps in the development of the mature virus? Nagington and Home think that if the threads or tubules are nucleoprotein, as was suggested by Higashi for other pox viruses, their accumulation to form the nucleoid can result from assembly from smaller units in situ or by tubules being produced before they are coiled up. In none of their observations was an incomplete nuclear structure seen. Moreover tubules have been observed unconnected with recognizable virus a short distance from what appeared to be an early incomplete form of the virus, five hours after infection. To prepare a model, tubules can be coiled around a central core or coiled within a hollow mould, and both methods are compatible with the accumulation of electron dense material seen in sectioned material. The formation of apparently empty particles first does in fact suggest that they are produced in order to act as a mould.
Another large pox virus which differs somewhat from vaccinia is that of contagious pustular dermatitis (orf virus) and it also has been studied with the negative staining method by Nagington and Home. In the particles of this virus the tubular components observed in the vaccinia virus appear to be wound in a criss-cross pattern. It is difficult in this case to say whether the tubular structures should be described as capsids or capsomeres, nor is it possible to say just where the nucleic acid is located in relation to them.

As regards the diseases produced by the pox viruses, the skin is often attacked forming lesions which may proliferate. Generalized infection usually follows except in the case of vaccinia which, as is well known, produces a local infection after vaccination against smallpox. Generalized infection with vaccinia does, however, sometimes occur, especially in children, and Andrewes points out that this seems to be unusually common when the virus is accidentally transmitted to contacts of vaccinated persons or to laboratory workers, usually through the skin.
CHAPTER TEN

HERPESVIRUSES

This is a large group of medium-sized, DNA viruses, and one of the characteristics of the group is their sensitivity to ether. They develop within the cell nucleus but develop round the particles a second membrane while leaving the nucleus and passing into the cytoplasm. Members of the group include the viruses of Herpes simplex of man, B virus or Herpesvirus Simiae Pseudorabies, Varicella and Zoster, and two viruses isolated from horses were found by Plummer and Water-son to possess many characteristics of the Herpes group. One was isolated from a horse with catarrh and called the LK virus. Parallel investigations of equine abortion (rhinopneumonitis) virus revealed a marked similarity between the two viruses although they are serologically distinct. It is suggested that the virus of equine abortion should be equine Herpes virus I and the LK virus would be equine Herpes virus 2.

In this chapter, however, we are mainly concerned with the virus attacking man and known as Herpes simplex or Herpesvirus hominis according to Andrewes. The chief interest of this virus derives from the fact that it is a latent infection in man and when stimulated into virulence produces the well-known 'cold sore' usually on the lips.

Morphology and Structure

Electron microscope studies by Home and his colleagues, using the negative staining technique with
phosphotungstic acid, have given much new information on the morphology and structure of the Herpes-virus particle. Four types of particle have been described but these are not so diverse as appears at first sight being full or empty particles with or without an enclosing envelope or membrane. The outer coat of the virus particle, the capsid, is composed of 162 sub-units or capsomeres set in such a way as to form an ico-sahedron, or twenty-sided figure. With negative staining and high magnification it was possible to see that the capsomeres were elongated hollow prisms, some hexagonal in cross section and others pentagonal. In the empty particles from which the nucleic acid core is missing, the PTA penetrates into the centre making the elongated capsomeres stand out clearly in profile at the periphery of the virus so that their hollow form and precision of arrangement can be seen. From the micrographs Home estimated the number of capsomeres for the Herpes virus. Of these 162 capsomeres, 12 are pentagonal prisms and 150 are hexagonal prisms. To satisfy the packing arrangement in accordance with ico-sahedral symmetry, the 12 pentagonal prisms would have to be placed at the corners and the 150 hexagonal prisms located on the edges or faces of the particle. The actual diameter of the Herpesvirus particle is 100-150 μ. It is interesting to note that apparently similar hollow capsomeres are found in the protein coat surrounding the Tipula iridescent virus particle (see Chapter Seven).

Development

The development of the virus of Herpes simplex has been studied by Rose and Morgan and much of the following information is derived from their work. The
earliest effect on the infected cell consists of nuclear changes in which reticular material accumulates on the inner surface of the nuclear membrane and the nucleoli undergo fragmentation. The nuclei may also appear somewhat swollen and irregular in shape, the remainder of the nuclear material being granular and less dense than usual.

The next development is the appearance of the virus particles themselves; at this stage they consist of a central body about 40 mµ, in diameter surrounded by a single membrane about 90 mµ, in diameter. While still within the nucleus these particles acquire a second membrane approximately 130 mµ, in diameter and it is possible that this second membrane is the same as the envelope round the capsid shown by Home and his colleagues.

The construction of this envelope or second membrane round the particle as described by Rose and Morgan is a remarkable process apparently designed to avoid rupturing the nuclear membrane itself by the passage of the virus outside the nucleus. Reduplication of the nuclear membrane seems to occur in such a way that the completed particles, with their double membrane escape from the nucleus by a mechanism in which new nuclear membranes are laid down successively behind the virus as it passes into the cytoplasm. The fact that viral particles frequently occur between the multiple nuclear membranes is further evidence for this ingenious method of virus escape.

Once in the cytoplasm the virus particle seem to be enclosed in walled vacuoles whose limiting membranes are probably derived from the nucleus. Rupture of these vacuoles at the cell surface can result in extrusion of the virus without disruption of the cell. It seems as if the
Herpes virus once formed is only slowly released from the cells.

Latency

As we have mentioned previously Herpes virus is a good example of a latent infection in man. Certain individuals commonly develop the well-known 'cold sore' generally on the lips at intervals throughout life. It is thought that such individuals acquired infection with the Herpes simplex virus probably in infancy and the virus remains quiescent or latent until stimulated into activity. The stress factors which cause stimulation of the virus are very varied and may be exposure to ultraviolet light such as occurs in bright sunlight, emotional stress or the interpolation of a second virus such as that of the common cold, hence 'cold sore'. The site of the latent virus in the body has not been ascertained.

Assay Methods

We have seen in Chapter One how the virus of tobacco mosaic may be assayed by counting the foci of virus multiplication (local lesions) which developed on the inoculated leaves of Nicotiana glutinosa. Then in Chapter Eight a method of assay for the animal viruses, developed by Dulbecco and his colleagues, is described. This method essentially involves the infection of monolayers of susceptible cells and has been successfully applied to Herpesvirus by a number of authors. A second technique, developed by Cooper, has certain advantages over the monolayer method. He mixed his infected cells with a suspension of agar and then plated that out to form monolayers in a petri dish in which plaques eventually appear. Plaques 1-2 mm. in diameter are produced in two to three days, the time for maximum
production of plaques depending on the type of cell infected. Plates are fixed with formol/saline, stained with Leishman's stain, dried and the plaques counted with a hand lens.

Chemical Constitution

Some studies on the chemical composition of the virus of Herpes simplex and of another member of the Herpes-virus group, Pseudorabies, have been carried out by Ben-Porat and Kaplan. They show that both viruses contain DNA and that in each case the DNA has a similar base composition. Moreover both viruses occupy the same position in sucrose or cesium chloride-density gradients. This is a comparatively new centrifuge technique which is very useful for separating virus mixtures. Briefly, the method involves the use of artificial density gradients and is roughly as follows: in each centrifuge tube equal volumes of decreasing concentrations of sucrose or cesium chloride are layered successively. One thus obtains a variation in density from the top to the bottom of the tube. The virus solution is layered at the top, and the tubes are centrifuged. Each component of the solution then moves down the sucrose gradient which finally consists of a series of successive layers. After the required centrifugation time has elapsed, the centrifuge is stopped and the tubes sampled. In some cases the zones are clearly visible, but in other cases samples are taken at definite intervals.

The fact that the Herpes and pseudorabies viruses occupy the same position in the cesium chloride-density gradients indicates that both viruses are of the same size and density.

The content of DNA per virus particle has been calculated to be $7.7 \times 10^{-12}$ per $\mu g$. Ben-Porat and Kaplan
have found that there is a relatively high content of guanine and cytosine in the DNA of both viruses.

*Disease produced by Herpesvirus*

Infection with Herpesvirus often takes place early in life and causes an acute stomatitis. Persons so infected, in spite of developing antibodies, are subject to recurrent Herpes throughout life usually in the form of the 'cold sores' or 'fever blisters' previously referred to. Various stimuli such as the common cold, exposure to the sun's rays, or emotional disturbances induce the formation of these blisters.

Young children are more susceptible than adults and frequently become infected by contact with the cold sores on their mothers' lips. Epidemics of Herpes among children sometimes occur.

Included in the group of Herpesviruses is the well known varicella virus, and it may be of interest to include it here because of the confusion that once existed over the two diseases 'chicken pox' and 'shingles'. It used to be thought that persons who had been infected with chicken pox (Varicella) in childhood were immune from infection with 'shingles' (Herpes zoster). This is not the case, however, as these diseases are of two clinical types caused by the same virus. It is thought that, as with Herpes simplex, shingles or zoster is due to stimulation of the latent chicken pox or varicella virus from a previous infection. It is possible to incur varicella after contact with a case of zoster, and varicella has been produced experimentally in children by means of zoster material.
CHAPTER ELEVEN

THE MYXOVIRUSES

Morphology

This group includes about twenty RNA viruses; these are the true influenza viruses, Influenza A, B and C, swine influenza and fowl plague and the paramyxoviruses which include mumps and Newcastle disease (NDV) and parainfluenza 1, 2, 3 and 4. They consist of spheres and filaments and are readily destroyed by treatment with 20 per cent. ethyl ether. The earliest stages of virus formation take place in the nucleus of the cell, other parts being added later as the particle passes through the cytoplasm. The virus reaches its complete infective form only as it is being liberated from the cell surface. (See Andrewes, Advances in Virus Research, 9, 1963.)

The lack of uniformity of structure in the myxoviruses is in striking contrast to such viruses as Herpesvirus or poliovirus. Using the method of negative staining with phosphotungstic acid, Home and his co-workers have studied the structure of roughly spherical or elongated particles of the myxoviruses. They consider that there are three constant features; first every particle when observed in profile shows a well-defined outer structure or membrane approximately 100-150 Å thick. Secondly, throughout the majority of the particles is an inner component whose structure appears mottled or markedly whorled. Thirdly, the outer membrane is covered by a series of projections measuring 80-100 Å from the membrane surface, and having a centre to centre distance of
70-80 A (Fig. 8). These projections are confined to the virus particles and have never been observed around cell components of similar size. It is worth noting here that apparently similar projections have been observed covering the particles of a plant virus, one which attacks the potato plant in the U.S.A. and is known as the potato yellow dwarf virus. (See Chapter Four).

The most striking structure seen which forms the main part of the mumps virus particles appears as a coiled hollow tube with a regular periodicity of 50 A along the axis.

The helical structures forming the inner core of the mumps, and Newcastle disease (NDV) viruses are thought to contain 9.6 and 5.7 per cent. of RNA respectively. We have seen one morphological resemblance between the myxoviruses and one of the plant viruses in the mutual possession of projections round the periphery of the particle. An even more striking feature is the resemblance to tobacco mosaic virus where the RNA is deeply embedded in the protein in the form of a helix (Chapter One).

Home and his colleagues suggest that this essential RNA component is in the form of a loose helix and is wound up into a coated arrangement inside the outer membrane. The sub-unit arrangement forming the helix would determine the rigidity of this component, and, in the case of mumps, this appears to be a flexible structure similar to filaments found in another plant virus, that of sugar beet yellows.

In a study of another virus belonging to the myxovirus group, the Sendai virus, Home and Waterson have obtained evidence of the possible existence of a double helix which would produce a more rigid structure. The two
structures forming the double helical array could contain separate RNA chains. The inner helical structures seen in the electron micrographs also offer an explanation of the existence of relatively large virus particles with low RNA content, as well as for the irregular size and shape of this group of viruses.

\textbf{Development of Influenza Virus}

We have mentioned already in giving the characteristics of the myxoviruses that the earliest stages of virus formation take place in the cell nucleus and that the virus reaches its complete infective form only as it is being liberated from the cell surface.

Hoyle has suggested that in these stages of virus formation there are three types of extrusion from the cell membrane, a large round body and two kinds of elongated particles. The long particles break up into spheres and the round body which is apparently extensible contracts to much smaller size. The morphological evidence suggests that the virus particle consists partially of host cell contents; the outer membrane is thought to consist solely of host material, the inner contents of the particle may be a mixture of virus and cell and the core of RNA is of purely viral origin (Figs. 9, 10).

Some interesting work by Blough (Virology, 19, 112, 1963) suggests that the surface state of the host cell may be a critical factor in determining the shape of the myxo-virus particle. He examined the effect of three synthetic detergents and of the enzyme lipoxidase on the subsequent shape of the virus grown in the developing hen's egg. As a result many filamentous forms were produced, approximately one third of the particles examined were filamentous, and short filaments were prominent in all treated specimens. Long filaments with
terminal bodies and 'drumstick' forms were especially prominent in the eggs treated with the enzyme lipoxidase and with Lubrol, one of the three synthetic detergents. It seems likely that the helical nucleoprotein conforms to, rather than determines, the shape of the enclosing membrane.

**The Natural History of Swine Influenza**

The story of swine influenza as elucidated by Shope serves to emphasize three important phenomena in the study of viruses. First it is a good example of a latent virus infection, secondly it illustrates the rather alarming capacity of some viruses to adapt themselves to a great variety of host organism and thirdly it shows the existence of reservoirs of virus infection where the virus can lie dormant for a large part of the year.

Swine influenza exists in epidemic form each year in the middle western United States, generally from October to December, and is largely absent from this region during the remainder of the year. Once the disease has started in a drove of pigs it is highly contagious and there is no difficulty in explaining its spread.

There were, however, two phenomena in connexion with swine influenza which were difficult to explain. One was the complete disappearance of the infection during the first part of the year, and the second was its sudden and simultaneous development in a number of droves of pigs, so sudden that it gave the appearance of a miraculously rapid spread. Indeed, the whole phenomenon resembled a 'spontaneous generation' of the virus.

In endeavouring to discover where the virus lay hidden during the absence of the disease, Shope was first put on the right track by an apparent superstition, widely held by farmers in the American middle west, that the
earthworm had something to do with swine influenza. Now there are two facts connecting earthworms with pigs, one is that the latter are fond of eating earthworms and the second is that the earthworm is the intermediate host of a parasitic lung worm which spends part of its life-cycle in the worm and part in the lungs of the pig. Could this lung worm play any part in the natural history of the disease? To test this theory a number of pigs were inoculated with the virus and then killed on the third, fourth and fifth day respectively after the onset of the disease. Lungworms were taken from these pigs, mixed with pig faeces and placed in a barrel of soil, to which were added about 400 earthworms.

Five weeks later some of these earthworms were examined and found to be infested with lung worms. The next step was to find out if pigs would develop influenza after feeding on these earthworms; the experiment was duly carried out but the pigs remained healthy. Something was lacking, and further investigation showed that a 'stress factor' was needed to stimulate the masked virus into action. We have now an explanation of the second of the two unexplained phenomena, the sudden and simultaneous development of swine influenza, and we find this in the 'stress factor' just mentioned. The apparent miraculous spread of the virus was in fact a simultaneous activation or stimulation of the latent virus infection brought about by a stress factor in the shape of a spell of adverse weather.

In one such experiment the selected pig was fed seventeen earthworms containing lungworms in the third developmental stage carrying masked swine influenza virus. Forty-three days after this feeding it was exposed outdoors to inclement weather for 18.5 hours.
Four days later the animal came down with an illness typical of influenza and swine influenza virus was demonstrated in its respiratory tract. In the place of inclement weather as a means of 'triggering off' the latent infection in his original experiments with the pigs Shope found that intramuscular injections with suspension of the bacterium Haemophilus influenza suis, either living or killed, induced the development of influenza.

Against the disease of influenza much good work has been done in the development of vaccines, the great difficulty being the occurrence of so many different strains of the virus. In the U.S.A. in 1943 subcutaneous vaccination with concentrated inactivated virus which had been cultured in chick allantoic fluid was found to have an effectiveness of about 75 per cent. against epidemic influenza A. The effect of the vaccine began to be noticed in seven to ten days after the inoculation, when the antibodies were accumulating. In 1945 the same vaccine which contained both type A and type B influenza viruses was shown to be effective in preventing an epidemic of influenza B. However, in 1947, this same vaccine was ineffective in the face of another epidemic of what was called the A-prime strains.

In 1957 there appeared the Asian strain of influenza A virus which immediately flared up into a pandemic since it was antigenically different from all other known strains and found everywhere a population susceptible to infection.

When this new Asian strain of influenza virus was seen approaching, an intensive effort was made in the U.S.A. to provide a vaccine against it. Material for the start of vaccine studies was obtained in two months, and the preliminary results suggested a 40-75 per cent. protection.
In the period of August to December over 50 million doses of vaccine were distributed, a remarkable achievement.

It will be seen, therefore, how the epidemiology of human influenza depends on the immunological varieties of the virus, and how pandemics ensue when a new strain of the virus arises to which no previous immunity exists.
ELLERMAN and Bang, working on the disease of chicken leukosis in 1908, were the first to show the connexion between viruses and tumours; thereby they opened up a new field of inquiry and raised the vexed question of the virus etiology of malignancy. This discovery, like that of Iwanowski, sixteen years earlier passed almost unnoticed and it was followed in 1911 by Rous's discovery of the sarcoma in chickens, which is now known all over the world as the 'Rous sarcoma'; we shall refer to this again later.

Since these early days many virus-induced tumours have been discovered and no less than eighteen virus sarcomas of birds have been described. In addition to birds, carcinomas of virus origin have been found in mammals, perhaps the most important being Bittner's discovery of the transmissible agent in mammary cancer in mice. Other malignancies include carcinomas induced in domestic rabbits by the virus of the Shope papilloma of wild rabbits, the mouse leukaemias, shown by deHarven and Friend to be transmissible by a cell-free filtrate, the remarkable polyoma disease and a kidney tumour in frogs.

In spite of the long and ever-increasing list of virus-induced tumours in animals, there has been for years a strong opposition to the theory of a virus etiology of cancer, some workers refusing to use the word 'virus' in this connexion but preferring such terms as 'tumour-agent' or 'milk factor'.
It may be opportune, here, to examine the two chief reasons usually given by those who oppose this theory. The first is the suggestion that the agents isolated from tumours are not viruses and the second is the admitted fact that no virus has yet been discovered in, or at least shown to be the cause of, any human cancer, and many other tumours.

The suggestion that there is any fundamental difference between tumour viruses and viruses causing other diseases can easily be discounted. Howatson points out that a capacity for tumour production exists in viruses of many different types; indeed this property cuts right across the boundaries of current classification schemes. Further, the tumour-inducing viruses may be either DNA- or RNA-containing types; they vary considerably in sensitivity to heat and to ether; they include viruses of very different sizes and of different architectural features, and they have different modes and sites of development within the cells they infect. Moreover, the ability, under appropriate conditions, to induce the proliferation and even malignant transformation of cells is not rare among viruses, and it is not unlikely that this property is even more widespread than is recognized at present.

The mode of transmission, also, is similar; for example the polyoma virus, which produces tumours of many different types, is infectious for mice, rats, hamsters, rabbits and guinea pigs when injected into newborn animals, and can be recovered from tumour tissue in these animals. The virus of visceral lymphomatosis of fowls is a contagious tumour virus and its transmission by way of water and excreta dispels the idea that tumour viruses have unique and mysterious routes of transmission.
Recently there has been the announcement of a tumour-virus affecting children in the tropics which might be insect-borne, and we can include here by way of comparison the wound-tumour virus which produces tumours in plants and is carried by a species of sap-sucking insect, a leafhopper.

The fact that no virus has been demonstrated as yet in a human cancer does not necessarily mean that it is not present; it may be there but in insufficient quantity for detection, direct experimentation not being practicable. Howatson points out that in some virus-induced tumours, virus particles can be detected only with difficulty, if at all, in the proliferating cells. For example, in the sarcomata induced in hamsters by polyoma virus and in papillomatous skin growth, most of the abnormal proliferation occurs where virus cannot be detected. In these instances the continuing presence of the virus is apparently unnecessary for maintenance of the stimulus for abnormal proliferation. Howatson suggests that the viral nucleic acid may enter into an intimate relationship with the cell genome, and the virus particles are lost as morphological identifiable entities. A somewhat similar condition seems to exist with the Rous sarcoma virus where certain of the cells are non-virus producing. These cells cause tumours when injected into newly-hatched chicks, but many of these tumours do not contain detectable virus.

Before we describe some of the more interesting tumour viruses, the following remarks of Lwoff may be relevant:

In a virus-induced cancer, a normal cell is altered by an oncogenic (tumour-causing) virus. The malignant cell continues to grow and divide, and, considered by itself, is healthy. A cell is, however, not an independent unit, but is
a dependent part of an organism. An organism controls the growth and multiplication of the normal cell but not of the malignant one, which behaves as an independent unit. Its multiplication causes the death of the organism. The oncogenic virus, although it only modifies a cell, kills the organism and is therefore pathogenic.

DESCRIPTION OF SOME TUMOUR VIRUSES

*Human Warts*

It has long been known that certain types of human warts were contagious and that they are caused by a virus. The first attempts to demonstrate the presence of a virus in warts were made more than ten years ago by some American workers, Strauss and his colleagues. They showed some electron micrographs of particles, measuring about 52µm in diameter, obtained from preparations of human warts which they believed to be the causal virus.

More recently, workers at the Ontario Cancer Institute in Toronto, re-examined the question using the thin-section technique and negative staining for electron microscopy. Four common warts, clinically typical of verruca plana and verruca vulgaris were examined by the thin-sectioning method. In three of these, the nuclei of some cells in the epidermis, contained uniform particles of mean diameter 46µm. In shadow-cast preparations the diameter of the particles was estimated at 55µm. When examined after negative staining they appeared also to be 55 µm in diameter and showed the same surface structure and symmetry arrangement as with polyoma virus, i.e. forty-two cap-someres. The capsomeres had an estimated diameter of about 8µm and were separated from each other by a distance of 4µm.
There has been some criticism of the symmetry determination and the estimated number of capsomeres in both polyoma and wart virus but some more recent work by Howatson and Crawford seems to confirm the number of capsomeres as forty-two. They examined disrupted particles of wart virus and counted the capsomeres.

The development of the wart virus has been studied by Howatson and the following information is derived from his work.

It is interesting to find that no virus can be found in the proliferating cells but only in succeeding layers where the cells become progressively more degenerate. This is a similar situation to that occurring in the development of the Shope papilloma and polyoma viruses and bears out the possibility that with some tumour viruses the presence of the virus particles is not required to maintain proliferation of the cells once the carcinogenic change is effected.

The wart virus develops in the cell nucleus, being first observed in the cells of the stratum spinosum. The earliest recognizable virus particles occur in close association with the nucleolus which seems to play a part in the synthesis of the virus. Close packed aggregates of virus are found in the nuclei of the cells.

**Mouse Mammary Tumour Virus**

This virus, which used to be known as the Bittner agent after its discoverer, causes mammary cancer in mice; it is important as being the first virus known to cause a carcinoma in a mammal. Bittner showed that the genetic background and hormonal make-up of the mice also played a part in the formation of the mammary tumour. Thus, there are low cancer-incidence and high cancer-incidence strains;
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the virus is normally transmitted in the mother's milk, so that foster-nursing could alter the rate of tumour-incidence according to whichever foster-mother was used. Andervont carried out an experiment to show that the virus is actually transmitted to the offspring in the mother's milk. He removed the young mice from a high cancer-rate mother by Caesarian section thus preventing any absorption of milk. By substituting a low cancer-rate foster-mother he suppressed entirely the mammary cancer which, in the line of mice being used, had an incidence of 97 per cent. Conversely, when the virus was introduced into a cancer-free line the incidence of the disease rose to over 70 per cent.

Purified preparations of this virus have now been made and examined in the electron microscope by Lyons and Moore. The particles resemble those of influenza but are somewhat larger, measuring 100-200 m in diameter. Surface projections 10µm long were observed and some particles showed evidence of an inner eccentric dense region or nucleoid. The inner structure, however, has not yet been resolved, although there does not seem to be present the internal helix seen in the influenza virus particle.

Howatson has studied the development of the mammary tumour virus, and he considers the particles to be intermediate in size between the small intramuscular viruses, like that of the human wart, and the large pox type of virus. The virus contains RNA and forms by a budding process at the cell surface, a process more reminiscent of the influenza group of viruses and quite different from some of the other tumour viruses. There appear to be two types of particles, one of them being probably the mature infective particle as it is found exclusively in extracts with
tumour-inducing activity. Howatson describes the developmental stage as follows. The first indication of particle-formation is a protrusion of the double layered cell membrane with the appearance of a cap of dense material on its inner aspect. This is also double-layered, the innermost layer being much thicker and denser than the other. As the protrusion develops the cap extends becoming first hemispherical and then a complete sphere. At the same time a neck forms in the protruding cytoplasm; this narrows and pinches off, severing the connexion of the particle with the cell. This whole process is somewhat reminiscent of the development of the myxoviruses described in Chapter Eleven and serves to emphasize the fact that the tumour viruses do not form a class separable from viruses in general.

*The Polyoma Virus*

The discovery of this interesting virus has stimulated anew the search for viruses in human cancer; it is unique among tumour viruses because of the range of species it affects, the many types of tumour that it induces and the rapidity with which it acts. As we have mentioned already, the virus is infectious for mice, rats, hamsters, rabbits and guinea pigs when injected into newborn animals; it can be recovered from the tumour tissue in these animals.

The polyoma virus has been studied by, among others, Stewart and Eddy and they found that in 400 Swiss mice injected with the virus no less than seventeen different types of tumour developed. These included salivary gland and bone tumours, gastric carcinomas, kidney sarcomas, thyroid, sweat gland and lung carcinomas and many others.
The following sequence of events appears to occur in the development of the mouse tumours. The first change noted is a ballooning of some of the epithelial cells in certain organs; this results from an enlargement of the nucleus which participates in the replication of the virus. Because of the massive quantities of virus formed and the associated damage to the nucleus, these cells do not go into mitosis but are destroyed. Adjacent cells or perhaps even cells in other organs are stimulated into tumour formation. It is not known whether this is brought about by the virus directly or through the action of a by-product of the virus.

Polyoma virus is of the DNA type and it has been shown that DNA derived from polyoma virus will give rise to virus in mouse embryo tissue, thus indicating that the DNA alone carries infectivity. No virus production has been detected in preliminary experiments with viral nucleic acid treated with the enzyme deoxyri-bonuclease, which destroys the DNA.

The polyoma virus particle has been studied on the electron microscope with negative staining by a number of workers and the results are summarized as follows by Home. The particles appear spherical when examined by the shadowing technique. Nevertheless negative staining shows that the outer shell is probably composed of forty-two elongated angular capsomeres arranged in icosahedral symmetry. Such a shell can be constructed by placing twelve pentagonal prisms at the corners of an icosahedron and thirty hexagonal prisms on the thirty edges. In this case the twenty faces have no capsomeres of their own which helps to explain the nearly spherical appearance of the virus.

There is now information on the methods of purification of polyoma virus and on its nucleic acid.
Winocour obtained a high concentration through the use of tissue cultures made from the kidneys of suckling mice inoculated with the virus a few days after birth. The virus was then centrifuged and purified by means of the density gradient technique using cesium chloride. Two main bands of differing density were formed; one showed the ultraviolet absorption spectrum typical of a nucleoprotein and under the electron microscope consisted mainly of 'full' virus particles. The other band of differing density gave an ultraviolet absorption spectrum for a typical protein and under the electron microscope consisted of 'empty' particles or 'ghosts'. This makes an interesting comparison with a similar purification of a plant virus, that of turnip yellow mosaic, in which empty particles, named 'top component' and full particles, named 'bottom component' occurred. (See Chapter Two.) The amount of deoxyribosenucleic acid (DNA) in the full polyoma virus particle was estimated at 13.4 per cent.

*The Rons Sarcoma Virus*

This virus causes a sarcoma in chickens which was discovered in 1911 by Rous and is known throughout the world as the Rous Sarcoma. It was the first tumour shown to be induced by a virus; since then much information has been obtained on the various neoplasms of chickens which are generally classified as the chicken leukosis complex.

A good deal is now known about this virus, it has been purified by density gradient centrifugation; it has been subjected to chemical analysis and shown to be an RNA virus. The base composition has been determined. Under the electron microscope the virus shows no evident symmetry. When examined after mixing with
phosphotungstate at pH 7.4, Rous sarcoma virus resembled myxovirus particles. According to Dourmash-kin and Simons the particle was shown to be roughly spherical or oval and measured about 900 to 1,200 Å across. Some of the particles displayed surface projections similar to those found in the virus of influenza, whereas others had relatively smooth surfaces. Many of the particles were flattened or disrupted and there was a suggestion of some internal component which, however, does not appear helical as in the myxoviruses.

One of the interesting and somewhat alarming characteristics of viruses is their apparent ability to adapt themselves to all kinds of different types of organism. We have seen this in the account of swine influenza where the virus can accommodate itself to pigs, lung-worms and earthworms. When Peyton Rous first discovered the chicken sarcoma, he found it difficult to transmit the virus to chickens which were not of the particular inbred strain he was using. Later it became transmissible, not only to other strains of chickens but also to ducks. Now it has been reported by Munroe and Windle that tumours can be produced in primates by injection of the chicken sarcoma virus. The experiment was performed as follows; a suspension of a variant of Rous sarcoma was injected into four adult and eight newborn rhesus monkeys. Seven of the newborn monkeys developed tumours, three of which were diagnosed as fibrosarcomas, in two to six weeks; none of the adults had tumours after eleven weeks. This seems to be another instance of the susceptibility of the very young animal to virus infection; we have seen the same thing in the case of the polyoma virus and, of course, suckling mice are much used in animal virus studies. The presence of virus in two of the tumours was demonstrated by injecting
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Tumour suspensions into the chick wing-web where tumours subsequently appeared. If confirmed, this seems to be the first time that sarcomas have developed in primates after a virus has been injected.
SUGGESTED FURTHER READING


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