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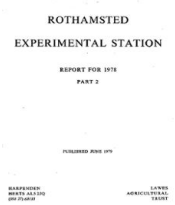

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Forty Years' Research on Plant Viruses at Rothamsted Experimental Station

B. KASSANIS

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Much of the work reported was done jointly with others and I take this opportunity to thank all my colleagues for their contributions and their friendliness during collaboration. To limit the size of this review their names have been omitted and no attempt has been made to connect my work with that of others except in a very few instances. A complete list of references to all my publications is provided in chronological order but the text is arranged in a natural order and the number of the relevant reference given in parenthesis.

Factors affecting susceptibility to infection

During the war, virus diseases of potatoes were studied in an effort to increase the food supply. By devising a method for testing susceptibility of potatoes, using aphids as vectors, a considerable difference was found in susceptibility among varieties of potatoes to potato virus Y and potato leaf roll virus, showing the importance of breeding for resistance varieties (13). It was also shown that plant breeders should examine the susceptibility of the new varieties not only to one strain of potato virus Y, but to a selection of strains because strains differ widely in virulence and the type of symptoms they cause (16). Sometimes, even within one variety there are lines that differ in their reaction to infection, e.g. the tobacco variety White Burley contains two distinct lines which react differently to strains of tobacco mosaic virus (TMV) (17).

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Carborundum is known to facilitate the transmission of viruses, but its effectiveness was shown to depend on its particle size. Celite was introduced as a useful alternative abrasive suitable for soft plants. Evidence was given that abrasives not only make wounds for virus to penetrate but also increase the susceptibility of the cells through some metabolic changes caused by the wound (11). Sugar-beet plants were infected for the first time with sugar-beet yellows virus, hitherto transmitted only by aphids, by using abrasives and young plants and keeping the plants for a period in the dark. These three factors increase susceptibility of plants to infection (21).

Fertiliser treatment that greatly influenced the growth of tobacco and potato plants in pots had little effect on the number that became infected with potato virus Y using aphids. But tobacco plants became more susceptible to mechanical inoculation with strains of TMV by the addition of N and P when used in amounts that increased growth, which suggests that conditions of maximum susceptibility approximate closely to those producing optimal growth (23).

It was known that keeping plants for a day or two in the dark made them more susceptible to infection with viruses. Keeping the plants at 36°C in the light for a similar period also increases their susceptibility (31). Attempts were made to find how darkness increases susceptibility. The susceptibility of plants increases directly with their water content suggesting that high osmotic pressure and turgor might help the establishment and multiplication of the virus (40). The same explanation might be true for plants kept at 36°C in humid atmosphere. On the other hand, exposure to about 50% CO_2 atmosphere for 15–60 min decreases the susceptibility of bean plants to infection with tobacco necrosis virus. The physiological changes responsible for the decrease are readily reversed by returning the plants to the air 4 h before inoculation. The decrease in susceptibility is found if the plants are exposed to CO_2 up to 4 h after inoculation but not later, suggesting that the effect is on the initiation of infection and not on the replication of the virus (9).

Saps from certain species of plants are known to prevent infection when mixed with the virus before inoculation. *Phytolacca esculenta* is one such plant which contains a powerful virus inhibitor. In 1948 the inhibitor was isolated and showed to be a protein, possibly a glycoprotein. It combines with TMV and, in salt-free solutions at pH values between their isoelectric points (pH 7 and 3·5 respectively), the two precipitate in the form of paracrystalline threads. Non-infective mixtures of the inhibitor and virus regain infectivity when diluted. It has been suggested that virus inhibition is caused through some physiological changes in the cell that affect its susceptibility, or through competition for the sites of infection (19).

The extent to which infection is reduced by inhibitory substances present in plants depends on the species of plants to which inoculations are made and not on the identity of the virus. The plant inhibitors are largely ineffective in preventing infection of the species which contain them (34). Apart from plant extracts many other substances can act as inhibitors of virus infection. Neutralisation of infectivity of viruses by homologous antisera was known and shown to be caused by antibodies other than the precipitating ones. Non-specific neutralisation by normal and heterologous sera can also be fairly high, especially with freshly prepared sera. The non-specific neutralising power falls rapidly on storing but inactivation of the serum complement is not responsible for the fall (6, 129).

Early stages of infection

When TMV is infiltrated into tobacco leaves, rubbed on to bean leaves (immune to TMV infection) or adsorbed to leaves of either plant by immersing them in the inoculum, more than half of the virus is inactivated and eventually uncoated and denatured over

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the next 24 h without causing infection. In fact the evidence is that only a negligible amount of virus enters the cells during inoculation relative to the amount adsorbed and strongly adhering to the leaf surface. Five minutes after immersing and washing the leaves, about 30% of the adsorbed virus is inactivated and 50–60% after 1 h. Electron microscopy showed that about 30% of the virus particles were shorter than complete particles, about 10% were completely uncoated in the first hour and 50–60% were completely uncoated in the next 24 h. The uncoating appeared to take place from one end. TMV treated with EDTA degrades a little more readily than untreated virus and it appears that inactivation and uncoating might take place in a hydrophobic environment by a physical disruption of bonds between the protein subunits and the RNA of the virus and that this is facilitated by chelating substances (128). It appears therefore that there is a protective mechanism for virus degradation at the surface of the cell wall.

After the discovery in 1956 by Gierer and Schramm that the nucleic acid (RNA) of TMV was the infective entity of the virus particle, it was used as a research tool. The infectivity of the RNA of TMV was about 1/100 that of the whole virus and it was therefore a great surprise when the RNA of tobacco necrosis virus was found to be highly infective and under some experimental conditions was as infective as the whole virus. I suggested that there was no intrinsic difference between the RNAs of the two viruses, but that the apparent difference in infectivity between them arises because the comparisons were made with whole virus, and as tobacco necrosis virus is much more unstable than TMV therefore is more likely to be inactivated before causing an infection (53).

Using tobacco necrosis virus, newly formed virus is detectable in the infected leaves 2–4 h sooner when the inoculum is RNA than when it is whole virus. The infective centres initiated by RNA develop increased resistance to ultraviolet radiation 2–3 h earlier than those initiated by whole virus. The necrotic local lesions appear over a period of time but those produced by RNA inoculum reach their final number sooner than those produced by whole viruses (53). Normally, with strains of tobacco necrosis virus, the resistance of virus in inoculated leaves to inactivation by ultraviolet light remains constant for about 2 h before increasing (lag period) but with the strain B, which is rather unstable, resistance to ultraviolet radiation decreases during the first hour before starting to increase (66). All these experiments suggest that before infection takes place the RNA is uncoated which causes a certain delay in the infection process. Similarly, TMV infection occurs sooner when the inoculum is virus RNA than when it is whole virus. In plants kept at 28°C newly formed virus becomes detectable 6–8 h after inoculation with virus RNA and 8–10 h after inoculation with whole virus. Also, infective centres initiated by virus RNA become resistant to hot water treatment (a 30 s dip in water at 50°C) 2–4 h sooner than those initiated by whole virus (52). Further detailed studies of early stages of infection using leaves of French beans and tobacco necrosis virus showed that newly formed free virus RNA could be detected about 4 h after inoculation, 2 h before complete virus (67).

Virus replication

Virus concentration. The virus concentration in plants depends on many factors one of which is the environment in which the plants are grown. Using tobacco plants, TMV concentration varies greatly with the mineral nutrition of the plants and is related to the plants' growth. Applications of N and P that stimulate vigorous plant growth also increase virus production, both per unit weight of leaf and more strikingly per plant. This may not be true with K which often stimulates plant growth but gives less virus per unit weight of leaf. Often virus is produced at the expense of normal proteins but this is not always so. On average about 1/3 of the total N occurs as virus but the range varies

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from 10 to 60%, depending on the fertiliser treatment and in one experiment reached 80% in plants that received a supplement of P but not N (24).

TMV reaches a higher concentration in inoculated tobacco leaves when they are detached and placed in water a day after inoculation than when they are left attached to the plant. The virus concentration is even higher (twice as much) when the leaves are placed in a solution of 10 g litre^{-1} sucrose and 0.2 g litre^{-1} calcium phosphate (32).

A considerable amount of work was done on the effect of high temperature (36°C) on virus multiplication but this will be described in a later section. In a detailed study of four different strains of TMV at low and high temperature some of the difference in concentration was found to be caused by the defective behaviour of the virus protein (96).

Cell inclusions. Before the nature of viruses was established virus workers used to pay special attention to characteristic 'inclusion bodies' found in cells of virus-infected plants. There were many speculations as to their origin or significance. Tobacco etch virus was shown to produce characteristic intranuclear inclusions. This was the first record of intranuclear inclusion in virus-infected plants although they were known to be common in animal infections (1). A number of other new forms of cytoplasmic inclusion were observed in 1940 in plants infected with different strains of TMV. These were mostly fibrous: spindle-shaped bodies, short needle-like fibres and extremely long coiled fibrous forms in figures of eight. It was also shown that amorphous spherical inclusions become crystalline with time. The new forms were attributed to the very fine weather of summer 1940 (4).

In later years this interest was extended to studies combining light microscopy with electron microscopy. Hyaline and granular inclusions were seen in cells infected with tobacco necrosis virus and its satellite (90). The Ni 118 nitrous acid mutant of TMV was found to produce numerous inclusions in cells of tobacco plants kept at 36°C and only a few in those kept at 20°C. At 36°C the virus protein is defective and it is not able to coat the virus RNA; it appears that the inclusions consist of this protein (98). Cellular inclusions were also found in infections with mutant PM_2, another nitrous acid mutant of TMV. Two variants of the mutant were investigated, one from Germany producing soluble but defective virus protein and one from England producing insoluble defective virus protein. Plants infected with the German variant contained long fibres usually twisted in the form of a figure eight and those with the English variant contained amorphous inclusions like those in Ni 118 infections. The German variant also showed open flexuous helical structures of the aggregated coat protein in negatively stained preparations of sap when examined in the electron microscope (101).

Further, electron microscopy of tobacco plants infected with potato mop-top virus showed inclusions very similar to those found with TMV, an interesting discovery in view of the serological relationships shown to exist between these two otherwise different viruses (103).

Inhibition of virus replication. Commoner and Mercer in 1951 found that thiouracil inhibited the replications of TMV in inoculated leaf discs and that this inhibition was reversed by adding excess of uracil to the solution in which the discs were floated. Their observation looked very promising, but when the work was repeated at Rothamsted by spraying the entire plant, although the claim was found to be accurate, the effect of thiouracil on plant growth was catastrophic. New growth of the plant was completely inhibited and generally the entire plant suffered severely. It appears that the inhibition of virus replication is linked to a general disturbance of the RNA metabolism of the plant by thiouracil rather than to the specific replacement of uracil by thiouracil in the virus RNA which was suggested as the cause of inhibition. This view is supported by the

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fact that thiouracil did not inhibit replication of tomato bushy stunt virus in French beans (33). Three years later thiouracil was used to free tobacco callus tissue from infection with potato virus Y by considerably disturbing the growth of the tissue for a period during which the virus was inactivated. This exercise found no practical application. The work with thiouracil and other base analogues by Matthews and others has shown how difficult it is to find an effective chemotherapeutic method to control plant viruses (47).

A natural defensive system against viruses (Effect of polyacrylic acid)

After virus infection, animal tissues become more resistant to reinfection and the increased resistance is caused by interferon, a protein produced during infection. Interferon is also produced and resistance to infection develops when animal tissues are injected with various synthetic polyanions one of which is polyacrylic acid. When this is injected into plants they also develop resistance to virus infection. Polyacrylic acid injected into the intracellular spaces of leaves of tobacco cv. Xanthi-nc induces complete resistance to infection with TMV and tobacco necrosis virus but only partial resistance to potato virus X, suggesting that the plant reacts better against a virus that causes a hypersensitive reaction, i.e. a virus that produces local lesions but not systemic infection. The resistance is highest when the injection is made 2–3 days before inoculation. The smaller the molecular weight of the polymer the better is the resistance to infection; this and other results suggest that the cell wall is a barrier to penetration by the chemical. The negative charges of the polymer seem to be essential for the plant to develop resistance as polyacrylamide, of the same molecular weight, fails to induce resistance. Three new proteins develop in the resistant plant, resembling the production of interferon in animal cells. The three proteins produced after injection of leaves of tobacco cv. Xanthi-nc with polyacrylic acid co-electrophorese with three of the four proteins produced in TMV-infected leaves. Resistance and proteins disappear when the plants are kept for a period at 32°C (111).

As with animals, plants infected with one virus become resistant to infection with a second different virus, but only when the second virus is one that causes hypersensitive reaction. This parallels the effects of polyacrylic acid. Leaves of tobacco plants cv. Xanthi-nc inoculated, or systemically infected with potato virus Y, cucumber mosaic virus, potato virus X, potato aucuba mosaic or alfalfa mosaic virus show varying degrees of resistance to infection with TMV which induces a hypersensitive reaction in this cultivar of tobacco. The resistance is correlated with the appearance of three new proteins similar to those found in polyacrylic acid injected leaves. Similarly, resistance and protein disappear when infected plants are kept for 2 days at 32°C (112).

Actinomycin D inhibits the resistance of tobacco to TMV that is induced by polyacrylic acid or by infection with a virus and also the production of the three to four proteins. Similar inhibition of resistance and interferon production is known to occur in animal tissues. This implies that resistance and protein production are dependent on DNA coded information of the cell (115).

Injection of chemicals into plants is not a practical method of application and therefore sprays were tried. Low molecular weight polyacrylic acids (3500 and 1700 mol. wt) were sprayed on to leaves and found to cause resistance to infection in tobacco cv. Xanthi-nc. Again the resistance is against viruses like TMV that cause a hypersensitive reaction and it is less effective with potato virus X or potato virus Y that become systemic in this cultivar. However, this work on natural resistance mechanisms in plants has only just begun and it is possible that more effective chemicals will be found that will stimulate plants to produce adequate resistance even against viruses that become systemic (116).

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With animal tissue interferon produced as a result of infection has been isolated and shown to cause resistance when injected into healthy tissues. However, the proteins produced during infection in plants do not induce resistance to virus infection when added to a culture of tobacco protoplasts, but it is too early to draw a final conclusion (127, 130).

Properties of new or lesser known viruses

New viruses have been found and their properties studied and viruses that were not very well known have been further characterised. Often the intention was not only to characterise the viruses but to use them in furthering our understanding of basic aspects of virology. The following table lists these viruses and gives relevant references.

The two most exciting projects connected with the discovery of new viruses concerned the study of satellite virus, which will be described in a later section, and of carnation latent virus which led to the discovery of two very important and widespread viruses, potato viruses S and M. While studying viruses affecting commercial carnations, three different viruses were readily identified by the characteristic symptoms caused on Sweet William plants (*Dianthus barbatus*) and some other hosts. Attempts to identify these viruses serologically at first gave conflicting and confusing results. Eventually the presence of a latent virus was suspected and its presence confirmed by examining serologically healthy looking Sweet William test plants that had been inoculated with heated sap from a plant infected with the virus mixture.

TABLE 1

Name of virus	Host plant	Reference
Tobacco etch virus	Solanaceae	2
Dandelion yellow mosaic virus	Dandelion, lettuce	8, 15
Tobacco necrosis virus	Tulip and wide range of hosts	20, 35, 64, 66, 91, 125
Potato paracrinkle virus	Potato cv. King Edward	25, 57, 74
Potato viruses M and S	Potato	41
Broad bean mottle virus	Broad bean	28
Carnation latent virus	Carnation	36, 38, 39
Carnation ring-spot virus	Carnation	39
Carnation mottle virus	Carnation	39
Carnation vein mottle virus	Carnation	39
Tobacco wilt virus	*Solanum jasminoides*	50
Datura necrosis virus	*Solanum jasminoides*	50
Barley stripe mosaic virus	Barley	51
Satellite virus	Tobacco and other hosts	61, 89, 92
Plum pox virus	Plum and other trees	77
Dolichos enation or sunn-hemp mosaic virus	Legumes	82, 118
Potato mot-top virus	Potato	102
Potato aucuba mosaic virus	Potato	56, 104
Beet yellows virus	Sugar beet	121, 122
Beet cryptic virus	Sugar beet	124, 126

The latent virus causes no symptoms at all. It has slightly flexuous, filamentous particles about 700 nm long and so resembled potato paracrinkle virus, with which I was familiar. It was interesting to discover that the two viruses were serologically related. All potato plants of the cv. King Edward at that time contained potato paracrinkle virus, and all the King Edward plants tested reacted with the antiserum to carnation latent virus. My curiosity motivated me into testing other potato cultivars thought to be healthy. The first one tested was a plant of the cv. Arran Victory, a cultivar which reacts with a crippling disease when grafted with a scion taken from a King Edward plant. It was astonishing to find that Arran Victory plants also reacted with the antiserum to

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carnation latent virus, as did plants of a dozen other cultivars obtained from Scotland and certified as healthy. Electron microscopical examination of sap showed that all these so-called healthy plants contained the same filamentous particles found in carnation and King Edward plants and reacted with the antiserum to carnation latent virus. About the same time, workers in Holland independently found a new virus which was widespread in healthy looking potato plants. This was the discovery of the two serotypes, potato viruses S and M (36, 38, 39).

Serological relationships among viruses

Serology was consistently used in estimating virus concentration, characterising or identifying viruses and determining relationships among viruses. Although I have not been involved in the classification of viruses, I have contributed in grouping viruses serologically. Two classes of relationship were distinguished: one, where the viruses differ slightly serologically and cross absorption of an antiserum with a virus removes most of the antibodies, and the other, between viruses that differ considerably in their antigenic determinants and where cross absorption reduces the serological titre of the absorbed antiserum only slightly or not at all. To facilitate nomenclature and classification of viruses the term 'serotype' was proposed for the second type of relationship, and the term 'strain' was reserved to denote close serological relationship. For example, potato viruses S and M and carnation latent virus are three serotypes while potato virus M and potato paracrinkle virus are strains (41, 57). Other viruses tested serologically and classified as serotypes were: barley stripe mosaic virus and lychnis ringspot virus (63), TMV, cucumber green mottle mosaic virus and sunn-hemp mosaic virus (83), TMV and potato mop-top virus (102).

A detailed serological investigation was made of tobacco necrosis viruses isolated in England and America and classified into two groups of serotypes A and D. In each serotype there were several strains. Bawden placed under the name of tobacco necrosis virus serologically unrelated viruses that produced necrotic local lesions in tobacco and French bean plants. He was probably forced to take this unusual step because one of the virus cultures, namely the 'Rothamsted culture' contained satellite virus which was later shown to be serologically unrelated to tobacco necrosis virus. The name tobacco necrosis virus was redefined to include only serologically related viruses (64).

Antisera prepared against different strains or serotypes of tobacco necrosis virus and satellite virus were most specific in precipitation tube tests when rabbits were bled after a single intravenous injection. In Ouchterlony tests, antisera remain equally specific after further injections, including one intramuscular injection showing that the Ouchterlony method is better in unravelling serological differences. However, the two methods agreed in indicating that the eight strains of tobacco necrosis virus tested were divided into the above two serotype groups A and D. In the same criterion three satellite viruses tested differed sufficiently antigenically to be classified as serotypes (93, 89).

Defective viruses

Inoculum from a single lesion of tobacco necrosis virus usually produces numerous lesions when inoculated on a test French bean plant, but occasionally such inocula give only one to five lesions. This could not be explained until it was realised that the virus in some of the single lesion inocula probably existed as free RNA and was therefore inactivated by the ribonuclease of the sap during extraction and inoculation. When these unusual lesions were extracted in water-saturated phenol or in 0·5M borax pH 9·2 the inocula were 10–100 times more infective than if extracted in water because the ribo-

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nuclease was rendered inactive. Such isolates were named 'unstable variants'. About 1 in 20 lesions formed by infective sap or purified preparations contain unstable variants. Therefore 5% of the tobacco necrosis virus particles produced during multiplication are defective mutants, i.e. are unable when multiplying in isolation to produce virus coat protein. In a mixed population, however, these mutants are coated by the protein produced by the normal virus (62).

Highly infective extracts of unstable variants of tobacco necrosis virus were prepared by grinding leaves in 0·06M pH 8 phosphate buffer containing bentonite and then removing the bentonite by centrifuging. Using such extracts more experimental evidence was thus accumulated to show that unstable variants were free virus RNA especially as they sedimented in a sucrose density gradient as virus RNA (67).

From a strain of dolichos enation mosaic virus (a serotype of TMV) an isolate was obtained which was extremely defective. At 20°C plants infected with it produced some particles without RNA. Purified preparations adjusted to pH 8 consisted mainly of discs, virus protein, some free virus RNA and very few infective rods of normal length. The isolate is thermophilic because infected plants kept at 36°C produce virus particles which do not differ in appearance from the parent strain. The parent strain was interesting because although it produced mostly normal length rods, it also produced particles 40 nm long in a concentration sufficient to form a peak when the purified virus was centrifuged in the analytical centrifuge. This short particle was later shown by others to be able to code for virus protein (82).

Defective mutants were also isolated, by the single lesion method, from the type strain of TMV. These mutants usually produce chlorotic or ringspot type symptoms on tobacco and are difficult to transmit unless carborundum is added to the inoculum. Their concentration is less than 0·1 μg of virus ml^{-1} of sap instead of the usual 2 mg ml^{-1} of the type strain. Phenol extracts from infected leaves are a little more infective than extracts in buffer suggesting that possibly the virus RNA is not well protected by the protein. In the electron microscope concentrated preparations show virus particles which are usually broken or appear inadequately assembled. These mutants appear to multiply very poorly, but their infectivity improved when inoculated together with the type strain, possibly because of phenotypic mixing (88).

PM_2, the nitrous acid mutant of TMV, produces defective virus protein at low and high temperatures (20–36°C) while Ni 118 does so only at high temperatures. However, the defectiveness of these two TMV mutants does not affect the rate of replication and concentration of their virus RNA as compared with the type strain. As the RNA is not protected by the virus protein, it degrades with time. By constrast the thermophilic isolate TC of TMV produces defective protein at 20°C and good protein and perfect particles at 36°C (96).

The knowledge accumulated from studying defective viruses was helpful in the purification and characterisation of potato mop-top virus which is in a way defective because it produces virus particles of varied length most of which uncoil from one end (102).

Carriers of virus infection

Carnation latent virus and its relationship to other potato viruses that are latent in many potato cultivars have been described already. Here I shall relate the story of the potato cv. King Edward, possibly one of the most interesting virus carriers. In the late 1920s N. R. Salaman found that when scions from apparently healthy King Edward potato were grafted on apparently healthy Arran Victory stocks a crippling disease developed in the Arran Victory stocks which he named potato paracrinkle virus. He failed to transmit this virus by mechanical inoculation to potato or other plant species. Various

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other workers confirmed that grafting Arran Victory with scions from King Edward plants always produced paracrinkle disease. It was therefore thought to be the perfect carrier, unharmed by the virus, and to be only of academic interest because it had no natural method of spread. Paracrinkle virus figured prominently in discussions on the origin of viruses and Darlington put forward the theory that there was no essential difference between viruses and plasmogenes (hypothetical units that determine cytoplasmic inheritance). Apparently what was a stable and presumably useful cell constituent in one genotype could act as a destructive agent (virus) in another. This theory was demolished when potato paracrinkle was seen in the electron microscope consisting of long slightly flexuous rods and the virus was transmitted by mechanical inoculation to tomato plants (25). Later, when a virus-free clone of the King Edward potato was produced it was shown that it was not the perfect carrier because the feathery appearance of the top leaves, characteristic of the infected clones, was absent from the healthy clone which had flat leaves (57).

An interesting carrier is *Primula obconica* inoculated with tobacco necrosis virus. The virus multiplies without causing any symptoms although in any other host that has been tried it produces necrotic local lesions. Even more interesting is the fact that the virus is localised in the infected centres showing that necrosis is not necessary for the localisation of the virus (14).

My last research was on sugar beet which is another carrier that may turn out to be as important in agriculture as the King Edward potato. Most sugar beet and the wild and cultivated varieties of *Beta vulgaris* contain a spherical virus without normally showing any symptoms. In all the varieties of sugar beet that have been examined 90% of the individual plants contain the virus which has been named beet cryptic virus (124). Selected healthy sugar-beet plants were propagated vegetatively and used for cross-pollination with infected plants. The virus was transmitted by both pollen and ovule but not by mechanical inoculation. A preliminary experiment comparing the growth of healthy and infected plants has shown that virus reduced the growth of the plant somewhat. So the virus may turn out to have economic effects and because sugar beet and other *Beta* plants are grown world wide the problem is likely to be important (126).

Interaction between viruses

Antagonism and symbiosis. At the beginning of my work and through my entire career I have been extremely interested in how viruses interact when present in the same host. The first interaction phenomenon was described in my Ph.D. thesis; it involved tobacco severe etch, potato virus Y and henbane mosaic virus. Plants infected with the first were protected from infection with either of the other two viruses suggesting some relationship between the three viruses. Although at the time no serological relationship was found, many years later, with improved technique the three viruses were found to be serotypes. However, what is unique about the interaction is that tobacco severe etch virus can suppress either of the other two viruses when plants are infected with mixed inoculum and even supplant them in tissue already infected. Similarly henbane mosaic virus suppresses and supplants potato virus Y (2). By contrast the multiplication of cucumber mosaic virus is unaffected by any of the three viruses (10).

Serologically related strains are antagonistic in their interaction; a plant infected with one strain is usually protected completely or to a large degree (depending on the closeness of the relationship) against infection with another strain of the same virus. The first outstanding exception was that between potato virus Y or C and the serologically related strain, tobacco veinal necrosis virus. Tobacco veinal necrosis virus does not protect *Nicotiana glutinosa* or potato plants against infection with potato virus Y or C and

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tobacco and *N. glutinosa* infected with potato virus Y or C can be infected with tobacco veinal necrosis virus (27).

Viruses that are only slightly related serologically do not usually interfere with one another and multiply unhindered in the same plant as has been found with potato virus S and potato paracrinkle virus (potato virus M) which are serotypes (41). Therefore, the antagonistic reaction described above between tobacco severe etch, potato virus Y and henbane mosaic viruses, which are serotypes, is unusual.

Phenotypic mixing (genomic masking). Under certain circumstances the RNA of one virus can be reconstituted with the protein of another, when the viruses are multiplying in the same plant. It has been mentioned earlier that tobacco necrosis virus produces unstable variants (mutants) that are coated with the protein produced by the virus normal particles (62). Phenotypic mixing was demonstrated also with the PM_2 nitrous acid mutant of TMV and sunn-hemp mosaic virus, a serotype of TMV. PM_2 multiplies in tobacco over the whole range of temperature (20–35°C) producing a defective protein that is not able to coat the RNA which therefore remains unstable. Sunn-hemp mosaic virus can multiply in tobacco only at 35°C. In mixed infections at 35°C the RNA of PM_2 is coated with the protein of sunn-hemp mosaic virus and becomes stable (94). Similar phenotypic mixing takes place between Ni 118, a nitrous acid mutant of TMV and sunn-hemp mosaic virus when inoculated together in tobacco plants kept at 35°C (97). Further work on phenotypic mixing showed that it is possible between strains or serotypes but not between two serologically distinct viruses (100).

Genetic recombination. In a review article on interactions of viruses in plants (68) evidence by others for genetic recombination was discussed and regarded as insufficient. The results of the experiments to support recombination could be explained by selection from mixtures or by mutation. It is reasonable to accept that all the genetical variation found in plant viruses can be explained by mutation, the frequency of which is sometimes extraordinarily high, e.g. the mutation rate of tobacco necrosis virus in producing unstable variants, as mentioned above, is 5%. Considering the number of particles present in a plant and the fact that the infection lasts during the entire life of the plant, mutation and selection brought about by changes in the environment could account for all the changes needed to explain the evolution of viruses. Furthermore with plant viruses the multicomponent system is widespread and variation can occur by mixing the components of closely related strains.

Although mutation is widespread I did not believe the extraordinary degree of mutation which Bawden claimed to take place when he described the change from one serotype to another. He claimed that when French beans were inoculated with the type strain of TMV it changed eventually into the serotype sunn-hemp mosaic virus and this was changed back to the original type strain when the inoculum was transferred into tobacco. The protein coat of these two serotypes differs by 96 amino acids and numerous mutations would be needed for the change to occur. When the work was repeated using his inocula it was found that the change did not occur and the reported results apparently were caused by contamination of one virus by the other (118).

Satellitism and multicomponent systems. Bawden and Pirie worked extensively with tobacco necrosis viruses in the forties and described the group as containing serologically distinct viruses, all causing necrotic local lesions on French beans and tobacco plants. They were particularly interested in the 'Rothamsted culture' of tobacco necrosis virus because it contained particles of two sizes 17 and 26 nm in diameter both consisting of nucleoprotein. Finally they came to the conclusion that the small particle was a by-product of the multiplication of the large particle which was the virus.

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In 1959 a tobacco necrosis virus was isolated from the roots of a naturally infected tobacco plant, and taking the normal precautions, it was passed several times through single lesion isolations. When the virus was multiplied on tobacco and purified it was found to react with the antiserum to the 'Rothamsted culture' of tobacco necrosis virus but surprisingly contained only the large particle. It appeared therefore that Bawden and Pirie's conclusion was not the right explanation for the existence of the small particle. Their isolate of the 'Rothamsted culture' was passed through many single lesion isolations and it was found that some of the single lesion isolates contained the large particle alone, some both particles but none contained the small particle alone. It was concluded from these results that the large particle could exist independently without causing the production of small particles while the small particle could only exist in association with the large particle. Using purified mixtures of the two particles, the small one was separated from the large one by sucrose density gradient centrifugation and shown to be non-infective. However, when the purified non-infective small particle was added to the pure large particle inoculum and the mixture inoculated to tobacco plants the small particle multiplied in large amounts together with the large one. It was also found that the two particles were serologically unrelated and that therefore the two were genetically distinct. It was then that the idea was conceived that the small particle was a virus but because of its small size was deficient and needed the assistance of the large particle for replication (54, 58). Because of its dependence, the small virus was called 'satellite virus' and the name tobacco necrosis virus was left for the 'helper' or 'activator' large virus. The biological properties of satellite virus were studied using a special technique that provided an estimate of its concentration. The satellite virus has an inhibitory effect on the multiplication of tobacco necrosis virus when plants are inoculated with a mixture of the two viruses; the lesions formed by tobacco necrosis virus are smaller and fewer in number, the decrease depending on the ratio of the two viruses in the inoculum. Also, the concentration of tobacco necrosis virus produced in the leaf decreases as the amount of satellite virus added to the inoculum is increased.

The most unusual property of satellite virus is its size. It is the smallest plant virus known, having a mol. wt of $1{\cdot}9 \times 10^6$ and containing 20% RNA with a mol. wt of $0{\cdot}38 \times 10^6$. The size of the RNA of satellite virus gives the clue to its dependence; it is too small to contain all the information necessary for replication and has to rely on the helper virus RNA (61, 68, 78).

Of all the strains of tobacco necrosis virus tested only strain D does not help the replication of the original strain of satellite virus. Satellite virus has a high isoelectric point of pH 7. As tobacco necrosis virus is isoelectric at pH 4·5 there will be a range of pH values where the two viruses could combine. It was thought that this might be a survival feature evolved by satellite virus to be in the proximity of its helper virus and thus cause mixed infections. However, the charges associated with these two viruses are not sufficient for attraction. However, mutual attraction occurs between TMV, isoelectric at pH 3·5 and bromegrass mosaic virus which is isoelectric at pH 8.0. At pHs between 4.0 and 6.5 the two viruses precipitate when mixed in salt-free solution (72).

Another interesting property of the original strain of satellite virus is that it crystallises easily and also forms stable aggregates of 12 particles or multiples (84). Many years later another larger virus (a strain of radish mosaic virus) was found to form similar aggregates (108). As radish mosaic virus has a two-component system, with different density particles, the aggregates form an extraordinary multiple pattern when purified preparations containing aggregates are centrifuged to equilibrium in the analytical centrifuge (109).

Apart from the original strain of satellite virus others have been isolated and one obtained from America. Three serotypes were tested but their antigenic specificity was

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not influenced by the activator strain used to help their replication. Some strains of tobacco necrosis virus aid the replication of the original strain of satellite virus but not the American strain and others behave in the opposite way. The ability of different strains of tobacco necrosis virus to aid the replication of satellite virus does not seem to be correlated with their antigenic grouping but rather with their ability to infect certain plants better than others (89, 93).

The English 1 and 2 strains of satellite virus not only interact and interfere with the helper virus but do so with one another in mixed infections, and the degree of interference depends on the strain of the helper virus. The interference between strains 1 and 2 takes place in the first 2 h after inoculation suggesting that they compete for an early metabolite. There is no interference between strain 1 or 2 and the American strain of satellite virus (105).

The close association between satellite virus and its helper virus was confirmed by electron microscopical observations. In doubly infected plants whenever satellite virus particles were detected in a cell, particles of tobacco necrosis virus were always nearby (90).

Satellite virus is not unique to plant viruses. A very similar phenomenon was also discovered in animal viruses. A simian adenovirus is sometimes associated with the so-called adenosatellite virus, which in many respects behaves like the satellite virus of tobacco necrosis virus. In plant viruses, the work on satellite virus served as a model in the discovery of the multicomponent system, when certain viruses were found to have two or more particles differing in their RNA content, all of which have to be present for infection to take place. This is a widespread phenomenon especially among spherical viruses, one of which I described myself. Thus, purified preparations of the kale and turnip strains of radish mosaic virus contain top component (characterised in the analytical centrifuge) without RNA and middle and bottom components differing in the amount of RNA they contain. The middle and bottom components when separated from each other are non-infective but infectivity is restored when the two are inoculated as a mixture. The kale and turnip strains are closely related serologically and the middle component of one will cause infection with the bottom component of the other and vice versa. However, heterologous mixtures of one component of either of these two strains with the other component of radish mosaic virus are not infective; radish mosaic virus is less closely related serologically to the turnip and kale viruses than these strains are to each other (108).

Effect of heat on infection

The work on heat treatment started with an attempt to free potato tubers from potato paracrinkle virus, but instead, these were freed from potato leaf roll virus. Tubers can be kept at 37–40°C in a moist atmosphere for a considerable time with little loss in viability. Those infected with potato leaf roll virus become virus-free after 25 days at 37°C, but potato viruses Y and X were not eliminated (22, 26). This success led to a detailed study of the effect of elevated temperatures on infections of plants with viruses that are mechanically transmitted so that the loss of virus could be quantified and possibly conclusions drawn concerning the mechanism responsible for inactivation. Most plants survive periods at 36°C provided the atmosphere is humid. It has already been mentioned that plants placed at 36°C for 1 or 2 days before inoculation become more susceptible to infection. Similar treatment after inoculation decreases the number of infections as measured by number of local lesions formed, but the precise effect of post-inoculation treatment differs with different viruses. Tomato spotted wilt and tobacco mosaic virus multiply in plants kept at 36°C and the treatment reduces the local lesion numbers by 10–90% of the untreated control. These two viruses have large temperature coefficients of heat inactivation *in vitro*, but differ considerably in their thermal inacti-

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vation end points. By contrast tobacco necrosis, tomato bushy stunt and cucumber mosaic viruses, are affected much more by post-inoculation treatment, lesion formation being completely prevented after a day or two at 36°C because the viruses do not multiply at this temperature. All these viruses have high thermal inactivation end points *in vitro*, but have small temperature coefficients of heat inactivation. The extent of thermal inhibition depends not only on the length of treatment but also on the physiological condition of the plants (31).

This work led to the discovery of heat therapy of plant virus diseases (1952). Kunkel more than 15 years earlier had cured young trees infected with peach yellow and other fruit tree diseases by prolonged heat treatment, but the causative agent of the diseases was not known. It was thought that the agents were viruses because the diseases were transmitted by grafting but we now know the causative agents are mycoplasma-like organisms. At Rothamsted heat therapy was used to free plants from infection with tomato bushy stunt, carnation ringspot, cucumber mosaic, tomato aspermy and abutilon variegation viruses. The leaves formed while the infected plants were kept at 36°C were without symptoms and virus free. Most cuttings taken after 3 weeks' treatment gave rise to healthy plants. The treated plants usually developed symptoms after a subsequent period at 20°C, but some remained healthy. The same treatment failed to cure plants infected with tomato spotted wilt virus, potato virus X or TMV, although it decreased their virus content. It appears that the multiplication of some viruses, particularly those with small spherical particles, is inhibited at 36°C and virus already present is rapidly inactivated. The fact that young and vigorously growing plants are cured much more easily than old ones, suggests that inactivation is an active metabolic process of the plant and not a direct effect of heat on the virus. This view is supported by the fact that although tomato spotted wilt has one of the lowest thermal inactivation end points *in vitro*, plants infected with it are not cured by heat treatment. As a result of these experiments, I suggested that the virus content of infected cells reflects an equilibrium between synthesis and degradation of virus, and that as synthesis of some viruses is inhibited at 36°C continuation of the normal degradation process, possibly at an increased rate, leads to the curing of plants or parts of plants (37).

Heat therapy was first applied on a practical basis to free chrysanthemums of several varieties from aspermy virus, chrysanthemum stunt and ring pattern viruses. The size, colour and shape of the blooms improved when the plants were cured (44). Now, heat therapy is usually combined with excising the apical part of plant shoots or the apical meristem and has been used to free from virus infection many cultivars of vegetatively propagated plants that were 100% infected.

In addition to the practical aspects of heat inactivation, my interests were also directed to academic problems concerning the general behaviour of viruses at high temperatures in the plant or *in vitro*. Avirulent variants (showing no symptoms) were readily obtained from tobacco plants inoculated with normally virulent TMV (type strain) when the infected tobacco plants were kept at 36°C but not when they were kept at 20°C. The avirulent strains were thermophilic as they reached a higher concentration than the parent strain in plants kept at 36°C, in fact the concentration of the parent strain declined detectably when kept at this temperature. It appears that high temperature selects thermophilic strains resulting from random mutations which occur all the time (45). Dolichos enation mosaic virus is another thermophilic virus which replicates at a higher rate at 36 than at 20°C and also forms virus which is more infective per unit weight and has more uniform particles (82).

The replication of four different strains of TMV, including three that produce defective protein, were compared at 36 and 20°C. At 36°C all strains produce the same amount of virus RNA but it is about 1/10 of that produced at 20°C. The effect of high temperature

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on whole virus varies with strain. With the PM_2 and Ni 118 strains, which both produce defective protein at 36°C, the virus RNA deteriorates rapidly at this temperature while with the TC strain, which produces defective protein at 20°C, the virus RNA deteriorates rapidly at 20°C (96).

Detailed investigations of the replication and heat inactivation at 36 and 20°C were also made on tomato bushy stunt and bromegrass mosaic viruses, both spherical viruses. They behave totally differently from each other. Tomato bushy stunt virus rapidly inactivates in plants kept at 36°C while bromegrass mosaic virus produces as much virus at 36°C as it does at 20°C and this virus is equally infective. The infectivity of tomato bushy stunt virus is lost *in vitro* and *in vivo* before there is any apparent change in the physical properties of the virus particles or virus RNA but eventually the virus particles disappear from the plants. Bromegrass mosaic virus, although very stable in plants kept at 36°C, loses its infectivity *in vitro* at 36°C at pH 7·0 (87).

Detailed studies of the kinetics of heat inactivation *in vitro* were made with several strains of tobacco necrosis virus. Tobacco necrosis virus is inactivated at two different rates as though it consists of two components. At high temperatures the more heat-resistant component is only a small fraction of the total, but increases with decreasing temperature and at about 40°C is the only component detectable. The inactivation rates of the two components differ greatly from each other and increase with increase in temperature. The changing ratio of the two components and other properties of the strains show that the virus preparations are initially homogeneous and that the two components are produced by heating. The RNA of tobacco necrosis virus is inactivated similarly as whole virus, indicating that changes in the RNA are responsible for thermal inactivation. The kinetics of heat inactivation of tobacco necrosis virus resemble those of some animal viruses and phages (65). Inactivation of tobacco necrosis virus by ultra-violet radiation is also caused by changes in the RNA (73).

Vector transmission

Aphids. The work on the properties of tobacco etch virus began at about the time (autumn 1938) when Watson classified aphid transmitted viruses according to the way in which they were transmitted. Tobacco etch virus is a non-persistent virus, i.e. the vector becomes infective after briefly probing the infected plant but loses its infectivity after a short period of feeding on the healthy test plant. Another interesting feature of this type of virus is that the percentage transmission increases when the aphids are fasted for 1–4 h before infective feeding (3).

Although most non-persistent viruses are acquired by their vectors during short feeds on infective plants, dandelion yellow mosaic virus, which does not persist for long periods in its vector, behaves differently. Its vector requires at least 3 h acquisition feeding before it can transmit the virus and the amount of transmission increases with increasing acquisition feeding time. Also, pre-acquisition starving of the aphids does not increase transmission. Therefore, of the characteristic features of the viruses described by Watson as non-persistent, the only constant character is the length of time the vector remains infective, and on that basis dandelion yellow mosaic virus is a non-persistent virus (8, 15).

The degree of specificity that exists between viruses and their vectors was illustrated by the example of *Myzus ascolonicus* which for a long time was considered to be a variant of *M. persicae* because of their morphological similarity. It was shown, however, that biologically, as vectors of viruses, the two aphids differ greatly (12). Virus–vector specificity was also shown by the behaviour of potato viruses C and Y which are serologically closely related, but only potato virus Y is aphid-transmitted. These two viruses were

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used later in trying to understand the mechanism of transmission. Also, the efficiency of different aphid vectors in transmitting potato virus Y varies considerably so that with some vector species only an occasional aphid transmits (16).

A few years later potato leaf roll virus was used to gain experience with persistent viruses. The work was facilitated by using *Datura tatula* as a test plant, because it is more susceptible than potato and also symptoms develop more quickly. The shortest acquisition-feeding time in which the vector *M. persicae* becomes infective is 2 h but such an aphid does not infect a healthy plant in the first 2 h of test feeding. These are minimum times and the ability to transmit increases with increasing length of acquisition feeding. By contrast, aphids after many days on infected plants can infect healthy plants after only 15 min test feed and continue to cause infection for a long period, sometimes until the aphid dies (30).

In 1936 Clinch noted that potato aucuba mosaic virus was transmitted by aphids only from plants which were also infected with potato virus A. This observation was given little attention until 1961 when this was confirmed at Rothamsted using 12 different strains of aucuba mosaic virus. None of the strains was transmitted alone but all were transmitted, to varied degree, from mixed infections not only with potato virus A but also with potato virus Y. The presence of the helper viruses increases the concentration of potato aucuba mosaic virus but it was shown that this is not the explanation for the phenomenon. The helper and aided viruses were both transmitted in a non-persistent way. The presence of the helper virus might cause potato aucuba mosaic virus particles to aggregate among themselves or to the helper virus particles and thus form larger units that might attach more easily to the aphid stylet, but this was not true (56).

Watson later found that potato virus Y also aids the aphid transmission of potato virus C from mixed infections. However, later it was found that mixed infection is not necessary for the aid to take place. Aphids (*M. persicae*) that have been starved for 3 h and allowed to probe for 1–2 min on a plant infected with the helper virus can acquire either potato aucuba mosaic virus or potato virus C and subsequently transmit them as efficiently as when they have had access to mixed infections. But, the sequence of probing is important, as no transmission takes place if the aphids probe first on the aided virus and then on the helper virus. The interesting fact is that although potato viruses C and Y are closely related serologically and such help may not be unexpected, there is no serological relationship between potato virus Y and aucuba mosaic virus (95).

Apart from potato viruses Y and A other viruses belonging to the potato virus Y group also act as helpers, although they differ in the efficiency with which they help in transmitting potato aucuba mosaic virus and potato virus C. Although aphids transmit the aided virus best following feeding for only a brief period on the helper virus, they are still able to acquire and transmit the aided virus to some extent following feeding for 2 days on the helper virus source. Also, starving the aphids between the two feedings does not completely eliminate transmission.

Important progress in this study was made when it was found that the helper virus need not be infective; the aided viruses are transmitted as frequently when the helper virus is inactivated by exposing the source leaf to ultraviolet radiation as when it is not.

Research was facilitated when it was found that transmission could be made by feeding aphids through artificial membranes. Virus is not transmitted by aphids feeding through artificial membranes, on water extracts of leaves infected with the helper, the aided virus or the mixture. However, potato aucuba mosaic virus is acquired from an extract through a membrane by aphids previously fed on a potato virus Y infected leaf. Similarly, infective potato virus Y is acquired from a leaf extract through membrane by aphids previously fed on a potato virus Y infected leaf that has been irradiated with

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ultraviolet light to inactivate the virus. These experiments suggest that a helper component produced during infection is needed for transmission and that this component is inactive in water extracts of infected leaves (99). However, it was later found that the helper component is preserved when infected leaves are extracted in a buffer containing EDTA and DIECA and aphids are able to transmit potato virus Y after probing through membranes into these extracts. Also, aphids transmit potato virus Y after probing through membranes into purified virus provided it is mixed with the supernatant obtained by ultracentrifuging a fresh extract of infected leaves; the supernatant is not infective and aphids do not transmit the virus after probing into it. The aphids also transmit potato virus Y if they are allowed to probe first into the supernatant and then into purified virus always through membranes. (The reverse was not possible.) Supernatants from fresh extracts of potato virus Y infected plants also aided membrane acquisition of henbane mosaic, tobacco severe etch and potato aucuba mosaic viruses. The results show conclusively that the extracts contain a substance of small molecular weight, the helper component, that is necessary for aphid transmission (110, 114).

Extracts of the helper component were active only when freshly made but later it was found that Mg^{2+} stabilises the component which can then be concentrated using polyethyleneglycol precipitation. After this treatment its activity is retained for 2 days at 4°C and several months at −15°C. Activity is destroyed on incubation with pronase or trypsin or by heating for 5 min at 55°C but not by incubation with ribonuclease. Incubation with its own antiserum strongly inhibits the helper component activity, but antisera to potato virus Y, virus coat protein or inclusion protein have little effect. The helper component is therefore a protein and was found to have a mol. wt of 100 000–200 000 and to bind firmly to the aphid (123).

That transmission is helped by a substance that binds the virus to some part of the mouth parts of the aphid is also shown by work done with poly-L-ornithine (PLO). When PLO is mixed with TMV virus, aggregates are formed and aphids probing into such mixtures through membranes transmit the virus when given acquisition and inoculation accesses of 30 s and 2 min. The ratio of virus and PLO as well as the concentration of KCl included in the mixture, markedly affect the transmission rate. At certain ratios of these three components aphids also transmit potato virus X and tobacco rattle virus (viruses that like TMV are not normally aphid transmitted), but not potato virus Y. Sequential acquisition experiments in which the virus is transmitted only if the aphids probe first on the PLO and then on TMV suggest that PLO may act by binding TMV to receptor sites in aphids which then transmit in a non-persistent manner (119). Helper component and PLO both bind the virus to the aphid receptors but it is likely that the binding forces are chemical with the helper component and electrostatic with PLO.

***Olpidium brassicae* as a vector.** After Teakle's discovery in 1960 that tobacco necrosis virus is transmitted from root to root by the zoospores of the fungus *Olpidium brassicae*, extensive work was done at Rothamsted to understand the mechanism of transmission, using roots of Mung bean seedlings, growing in a modified Hoagland's solution. Transmissions are obtained with as little as 0·05 μg litre^{-1} virus inoculum, which is 20–100 times less inoculum than is needed for successful mechanical inoculation. A minimum of 10^5 zoospores ml^{-1} are needed for transmission. It was interesting to find that roots exposed to zoospores for 10 min and then washed are readily infected by tobacco necrosis virus when roots are immersed in virus during the first hour or two after zoospore attachments to the root cells. Immersing roots, inoculated with fungus and virus in water, at 50°C for 10 s kills the fungus but not the virus and by varying the interval between inoculation and heating it was found that the virus becomes established 2–3 h after

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inoculation. This shows that the fungus is needed only for the penetration of the virus but not for infection. The virus is not present inside the zoospores because *O. brassicae* naturally infected with virus can be freed from virus by simple dilution or by centrifugation at low speed. Virus transmission is prevented by adding antiserum to the zoospores that had already been exposed to virus (70). These results suggest that the zoospores help the virus to penetrate the root cells in a mechanical way while they themselves penetrate the cell wall using enzymes.

Work was also done to investigate the relationship between different *Olpidium* isolates and virus using several hosts and the two virus serotypes A and D. *Olpidium* 4 did not transmit either serotype to any host. *Olpidium* 3 transmitted both viruses more readily than *Olpidium* 1. Serotype D was usually transmitted more readily than A but *Olpidium* 1 transmitted A more readily than D to tobacco callus tissue. The virus may be transmitted to callus tissues quite readily, the fungus not only aiding infection by the virus but also establishing itself in the callus cells to form mature sporangia (69). Apparent differences in efficiency of transmission of the two serotypes are not correlated with host ability to support virus multiplication. Neither can specific transmission by *Olpidium* isolates be explained in terms of different susceptibility of the hosts to individual *Olpidium* isolates. Eventually it was found that some of the specificities are caused by responses of the host to the *Olpidium* which sometimes result in unsuitable conditions for virus infection. Virus infection in cress roots with *Olpidium* 3 can be inhibited by inoculating the roots later with *Olpidium* 4. This inhibition can be reversed by heating the doubly inoculated roots at 50°C for 10 s which inactivates the fungus but not the virus. Other *Olpidium* isolates that do not transmit serotype D to cress, although they do to other hosts, can also inhibit transmission by *Olpidium* 3. This cause of specificity might be widely present even with other vectors such as aphids. A potential vector may introduce into the cell enzymes or other substances together with the virus that might render the cell unsuitable for replication (75).

None of the four isolates of *Olpidium* tested transmitted the original strain of satellite virus even when associated with its activator tobacco necrosis virus. Two other strains of satellite virus have been found, however, that are transmitted each by a specific *Olpidium* isolate, naturally, together with the helper virus (86).

From the practical point of view the fungicide captan was shown to delay root infection by 2–4 weeks in naturally infected soil. However, when a large amount of virus is added to autoclaved soil captan does not prevent infection suggesting that in the presence of sufficient virus inoculum some infection takes place through abrasion of the roots by soil particles (66).

Tissue cultures and protoplasts

In 1954 I made one of my few trips abroad in order to familiarise myself with Morel's work on plant tissue cultures and to see if it could be applied in virus research. I was impressed by the method of excising apical meristems of plant shoots and growing them into plantlets in artificial media. It had been suggested that the meristematic region of the growing point of infected plants was virus free and that therefore virus-free stocks could be obtained from infected plants. The first useful application of the method was to obtain virus-free plants of King Edward and Arran Victory potato. In later years virus-free clones were produced of several other English and Scottish potato cultivars that have been 100% infected with potato viruses M, S, A or X (46, 80). The idea that the meristematic tissue is virus free is now known to be untrue. It has already been mentioned that freeing infected callus tissue from virus using thiouracil was brought about by the inactivation of the virus by the adverse conditions presented to the tissue (47). In the

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same way the virus in excised meristematic tissue is inactivated because the tissue suffers considerably during the early days after excision. Freeing the potato cv. King Edward from potato paracrinkle virus was a great benefit to agriculture because of the cultivar's popularity. The yield of the virus-free stock was 10% higher than the infected and it was calculated that during the sixties the annual gain from the increased yield, using the healthy stock, was about £2 million (60, 74).

Normal and conditioned tobacco callus tissue are infected with TMV only through mechanical injuries and not in any other way. The success of inoculation depends on the type of injury. Occasionally, tissues can be infected by contact with the inoculum but probably because some cells are naturally injured. As there is no organised vascular system in the tissues the virus spreads by moving from cell to cell at the rate of about 1 mm per week (48). Not much benefit was derived from studying the multiplication of TMV in tumorous tobacco tissues kept in different environments (43).

After Takebe and his co-workers developed a method for releasing protoplasts from the mesophyll of tobacco leaves and infecting them with TMV, I helped the Department of Botany of the University of Nottingham to repeat and confirm this work (106) and we extended the work to yeast protoplasts which we were also able to infect to a small extent with TMV (107). Takebe's two-step method of producing protoplasts was later simplified at Rothamsted. The release was obtained without shaking, by incubating tobacco leaves, with stripped epidermis, in a solution containing pectinase and cellulase. The incubation medium was also simplified, but Ca^{2+} was necessary for the stability and infection of protoplasts. Using this method as much as 0·1–0·5 mg of virus per 10^6 protoplasts is obtained, i.e. as much as in the plant (113). The incubation medium was simplified to the extent of using mannitol alone, with only antibiotics added to prevent contamination by microorganisms. However, most antibiotics chelate metals from the protoplast plasmolemma and this inhibits multiplication of TMV. The inhibition can be overcome by adding $CaCl_2$ or even better $MnCl_2$. Addition of $MnCl_2$ also inhibits contamination by yeast so that the only antibiotic needed in the incubation medium is one to inhibit bacteria (117).

The multiplication of TMV in protoplasts is also inhibited by rabbit blood serum when it is added to the incubation medium at dilutions up to 1/300. Five minutes' exposure of the protoplasts to the serum, even when followed by washing, is sufficient to inhibit virus multiplication. The serum components responsible for inhibition are not dialysable but they are heat-labile. Heated sera cause less inhibition as do purified serum globulin or albumin. Addition of $CaCl_2$ (but not $MnCl_2$) decreases the inhibition. It appears that serum is bactericidal and its action is to immobilise or chelate Ca^{2+}. The active part of the serum is probably one of the components of the complement (129).

As workers disagreed about the mechanism by which protoplasts are infected by virus, this was investigated. Tobacco protoplasts are readily infected with TMV by inoculating them with 1 $\mu g\ ml^{-1}$ TMV and 2 $\mu g\ ml^{-1}$ poly-L-ornithine (PLO). Infection is not reduced when the amount of TMV is decreased to 1/10 but no infection occurs when PLO is similarly decreased. However, if a mixture of 1 $\mu g\ ml^{-1}$ TMV and 2 $\mu g\ ml^{-1}$ PLO is first incubated for 10 min, it can then be diluted to 1/10 without decreasing infection. The reason for this was found to be that virus and PLO aggregate during incubation and that these aggregates, once formed, are still effective when diluted. The aggregates are infective even after washing them free from excess PLO. Examination of the surface of inoculated protoplasts in the scanning electron microscope showed wounds which heal in a few hours. The experimental evidence shows that TMV–PLO aggregates have a high concentration of PLO which causes wounds in the plasmolemma through which the virus enters and infects the protoplasts. The mechanism is the same for plants, callus tissues or protoplasts (120).

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