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THE RELATIONSHIPS BETWEEN LIQUID CRYSTALLINE PREPARATIONS OF CUCUMBER VIRUSES 3 AND 4 AND STRAINS OF TOBACCO MOSAIC VIRUS.

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FOUR viruses affecting cucumber plants have been described by Ainsworth (1935) as cucumber viruses 1, 2, 3 and 4. These fall into two pairs, the individuals of each pair possessing similar general properties. Viruses 1 and 2 are transmitted by aphids, are inactivated by 10 minutes' heating at 60° C., or by a few days' ageing in expressed sap, and are readily transmitted to members of the Solanaceæ and other families. Cucumber viruses 3 and 4 are apparently not transmitted by aphids, are not inactivated by 10 minutes' heating at 80° C., or by some months' ageing in expressed sap, and they have not been transmitted to any plants except members of the Cucurbitaceæ. A further fact which relates viruses 3 and 4 and separates them from 1 and 2 is that cucumber plants infected with virus 3 are resistant to further infection with virus 4, but are still quite susceptible to viruses 1 and 2. Viruses 3 and 4 are differentiated merely because they cause different symptoms. Cucumber plants infected with virus 3 show a general dark green mottle and the leaves

become somewhat blistered and deformed, while those infected with virus 4 show a bright yellow blotchy type of mottling and but little blistering or deformation.

Ainsworth's results suggest that the relationship between viruses 3 and 4 might be of the same type as that between the strains of tobacco mosaic virus, and they also show that the stability of the two viruses *in vitro* is of the same order as that of tobacco mosaic virus. As far as is known the recognized strains of tobacco mosaic virus and cucumber viruses 3 and 4 have no common host plants : we have been unable to infect cucumber plants with three different strains of tobacco mosaic virus ; other workers have found the members of the Cucurbitaceæ to be immune to tobacco mosaic virus, and, as already stated, Ainsworth found the host range of cucumber viruses 3 and 4 to be restricted to the Cucurbitaceæ. Comparative infection experiments with the different viruses therefore cannot be made, and it is impossible to determine whether they are sufficiently related to immunize plants against one another. The results presented in this paper show that in spite of the wide differences in the host ranges of the two groups of viruses they are fairly closely related. They have antigens in common, and from cucumber plants infected with viruses 3 and 4 we have isolated nucleoproteins infective at high dilutions (Bawden and Pirie, 1937a). These nucleoproteins have similar analytical figures and many properties similar to those previously isolated from solanaceous plants infected with strains of tobacco mosaic virus (Bawden and Pirie, 1937b). The differences at present noted between cucumber viruses 3 and 4 and strains of tobacco mosaic virus, however, are considerably greater than those noted between the individual strains of tobacco mosaic virus.

PREPARATION.

The general methods described for the purification of the strains of tobacco mosaic virus have been used for the cucumber viruses, but as the latter proved rather more difficult to isolate, certain modifications of the original method have been made. The final yields obtained have also been less, averaging from 0·2 to 0·3 g. per l. of expressed cucumber sap as compared with about 2 g. with tobacco mosaic virus. Cucumber viruses 3 and 4 precipitate from plant sap at around pH 4·8, whereas the strains of tobacco mosaic virus precipitate at around pH 3·4. This fact possibly accounts for some of the difficulty experienced in freeing the cucumber viruses from plant proteins by precipitation methods. It is also of interest in view of the different host ranges of the two groups of viruses ; for the pH value of expressed cucumber sap is between 7 and 8, whereas that of tobacco sap is between 5 and 6. Of the methods of preparation yet tried the following has proved most effective.

Cucumber plants are picked about a month after infection when they are showing most definite symptoms, minced in a meat mincer and the sap expressed through muslin. The mincing is easier and more sap is obtained if the leaves are first sprinkled with a dilute solution of sodium cyanide. This also has two other useful effects : it largely prevents the formation of highly coloured oxidation products which are difficult to remove from the final preparation, and it increases the yield of virus by raising the alkalinity of the extract. 0·5 to 1 g. of cyanide should be used for each kilogram of leaves. The expressed

sap is then heated to 70° C., and rapidly cooled. This treatment produces a flocculent precipitate, which aggregates quickly and is readily thrown down by centrifuging. The precipitate should be thoroughly washed in water and again centrifuged, for a certain amount of virus adheres to it.

The greenish-brown supernatant fluid is then brought to about pH 4·8 by the addition of HCl, and the precipitate produced is centrifuged off. The precipitate is suspended in water and dilute NaOH solution added to bring the pH to about 7·5, when the suspension is centrifuged. Sufficient ammonium sulphate is now added to the clarified supernatant to give a quarter saturated solution, and the precipitate formed is centrifuged off. This precipitate is taken up in water, centrifuged and the precipitate discarded ; the supernatant fluid is then again one quarter saturated with ammonium sulphate. The precipitate obtained at this stage of the preparation is usually greyish in colour, and shows a pronounced satin-like sheen similar to that shown by preparations of tobacco mosaic virus. When examined microscopically (preferably with dark-ground illumination) it is seen to be composed of fairly regularly shaped needles, similar to those which Stanley (1936), working with tobacco mosaic virus, described as crystals. Bernal and Fankuchen (1937), however, have now pointed out that these needles lack the regularity characteristic of true crystals, and that they are ordered in two dimensions only. The precipitate is centrifuged down, dissolved in water and the solution again centrifuged. It easily comes clear by transmitted light, although retaining an intense sheen by reflected light. This fluid is diluted somewhat, and is then brought to about pH 4·8 by the addition of $N/10$ HCl, when a precipitate with the sheen is again produced. This is thrown down and then washed several times by stirring up with water and repeated centrifuging. When the water used for washing is nearly free from sulphate, the precipitate is dissolved by the addition of dilute NaOH solution. At this stage in the preparation of the strains of tobacco mosaic virus it was noticed that the precipitation point with acid showed a definite shift, and the precipitate obtained at pH 3·4 from dilute salt solutions became soluble at this pH when salt-free. This shift is presumably similar to the rather smaller shift in the same direction that has been observed with certain other proteins (Adair and Adair, 1934 ; Smith, 1936). Most preparations of cucumber viruses 3 and 4 have not shown a shift of this type, but a few were found to sediment more easily at about pH 5·5 when salt-free than they did at pH 4·8. In the complete absence of salt the viruses are not readily sedimented by centrifuging at 3500 r.p.m.

At pH 6 preparations of cucumber viruses 3 and 4 are definitely more turbid than are those of tobacco mosaic virus of the same concentration, but the turbidity disappears at above pH 7. Neutral aqueous solutions of the material prepared in this way, especially if young cucumber plants are used as a source of the virus, are sometimes quite colourless, and if more concentrated than about 3 p.c. separate on standing into two liquid layers. More often, however, preparations at this stage are quite definitely coloured and do not layer. Such preparations can only with difficulty be fractionated further by more precipitations with acid and ammonium sulphate. All the material in the solutions is precipitated by these treatments apparently in the para-crystalline form. Such preparations can easily be purified further either by

incubation with trypsin or by high-speed centrifugation. When preparations have been incubated with about 0·2 p.c. trypsin at 38° C. and at pH 8 overnight they have always given colourless neutral solutions which separated into two layers, after two or three further precipitations with acid or ammonium sulphate. Alternatively, the material can be fractionated by centrifuging the neutral solutions at 16,000 r.p.m. This treatment sediments a transparent jelly, which dissolves in water to give a colourless solution that separates into two layers.

The layering phenomenon in purified preparations of cucumber viruses is similar to that previously described with the strains of tobacco mosaic virus (Bawden and Pirie, 1937b). It has not been studied in such detail with cucumber viruses 3 and 4, but as yet it has been observed at room temperature only in solutions containing more than 2·5 p.c. of solids. At 0° C. layers can separate from more dilute solutions. The upper layer is the more dilute and by transmitted light is faintly opalescent ; the lower layer may be quite clear by transmitted light but generally has an intense sheen by reflected light. When solutions are left undisturbed for some months the lower layer loses the sheen, but it regains it if it is shaken with the upper layer and then again allowed to layer ; similarly, if a sample of the lower layer is centrifuged at 16,000 r.p.m. for half an hour a layer of perfectly clear liquid crystalline solution appears in the middle of the tube between the sedimented jelly at the bottom and the very dilute solution remaining at the top. This clear solution shows no sheen. It seems, therefore, that the sheen of the bottom layer is a result of incomplete separation, and is caused by droplets of top-layer fluid suspended in it, and is not a necessary consequence of its liquid crystallinity. The upper layer is not spontaneously birefringent, but readily shows the phenomenon of anisotropy of flow, i. e. it becomes birefringent when agitated or when flowing. The jellies which sediment when neutral or acid solutions of cucumber viruses 3 and 4 are centrifuged at high speed are also birefringent.

The sheen and the phenomenon of anisotropy of flow suggest that these particles, like those of tobacco mosaic virus, are rod-shaped, and this has been confirmed by the X-ray measurements of Bernal and Fankuchen (1937).

ANALYSIS.

Dried preparations of cucumber viruses 3 and 4 closely resemble similarly treated preparations of strains of tobacco mosaic virus. When dried in the frozen state the material has a light open texture and is easily handled. After drying the material is still infective, but its ability to show the phenomenon of anisotropy of flow is much reduced and its serological activity is reduced to about one-half. The analytical figures obtained have varied slightly from preparation to preparation, but normally fall in the following ranges :

Carbon	50·00 to 51·0 p.c.	
Hydrogen	7·10 ,, 7·6 ,,	
Nitrogen	15·30 ,, 15·8 ,,	
Sulphur	0·00 ,, 0·6 ,,	
Phosphorus	0·55 ,, 0·6 ,,	
Carbohydrate	2·20 ,, 2·5 ,,	
Ash	1·00 ,, 2·0 ,,	

The phosphorus and carbohydrate contents were estimated by the methods previously described (Bawden and Pirie, 1937b).   The phosphorus and carbohydrate can be isolated as nucleic acid after these viruses have been inactivated by heating, in the same manner as previously described for the strains of tobacco mosaic virus.   The intact nucleic acid + protein complex resists attack by trypsin preparations containing nuclease, but when the complex has been disrupted by heat the components are readily attacked by this enzyme mixture.   Schmidt (1936) has found other nucleoproteins to behave in this way.

Further evidence that the nucleic acid and protein are combined and not merely mixed in the virus preparations, as Stanley states (1937a and b), was obtained by analysing the precipitates obtained by centrifuging the virus preparations at a high speed.   When solutions of the cucumber viruses are centrifuged at 16,000 r.p.m. at pH 8 and pH 3 birefringent jellies are deposited. After drying, both these have the same nucleic acid content as the material that is obtained by centrifuging at low speeds at the acid precipitation point. Stanley (1937b), in an attempt to show that the protein he had isolated was tobacco mosaic virus and not a mixture of protein and virus, centrifuged his products at a number of pH values on either side of the iso-electric point, and showed that the sedimentation rate of the virus (measured by infectivity tests) was the same as that of the protein.   From this he argues that the virus is not merely a contaminant adsorbed on to the protein, for if it were it should come off when the pH is sufficiently altered.   If this is a valid argument it should also have been possible to separate the nucleic acid from the protein in our experiments by altering the pH if the two components were merely mixed.

The only evidence against our conclusion that the isolated materials are nucleoproteins is in two of Stanley's statements.   Firstly (1936), he stated that his preparations of tobacco mosaic virus contained no phosphorus, and secondly (1937b), that, as usually prepared, they contain phosphorus and carbohydrate in the form of nucleic acid, but that this is inessential for virus activity and can be removed by prolonged alkaline dialysis.   Best (1936) has shown that the infectivity of tobacco mosaic virus preparations is reduced to one half by 12 hours' exposure at room temperature and pH 8·2.   Stanley has published no figures to show that the serological activity and infectivity of his virus preparations are unaffected by the treatments used to remove the phosphorus and carbohydrate.   In our experience those treatments which lower the phosphorus content to a level at which its detection becomes difficult, $i.\ e.$ 0·05 p.c. or lower, invariably lead to a corresponding decrease in the activity of the virus preparations.   The resulting products are still highly infective, $e.\ g.$ infections may be obtained with as little as 10^{-8} or 10^{-9}, but it seems reasonable to assume that these infections are brought about by the remaining, undecomposed nucleoprotein, and not by the dephosphorylated protein which constitutes the bulk of such material.

ACTIVITY AND SEROLOGICAL REACTIONS.

No host plants are known in which cucumber viruses 3 and 4 produce local lesions, and the quantitative methods used for determining the infectivity

of the tobacco mosaic virus preparations at various dilutions could not be employed. The infectivity of the purified cucumber viruses was therefore tested merely by rubbing the leaves of cucumber plants with 1 c.c. of solutions containing various amounts of nucleoprotein, and noting the greatest dilution at which systemic infections were obtained. The results of six tests of this type are shown in Table I, and it will be seen that the smallest amount required to produce infection has varied from 10^{-8} to 10^{-10} g.

TABLE I.—*Infectivity of Purified Preparations of Cucumber Viruses 3 and 4.*

Virus.	Dilution.			
	10^{-7}.	10^{-8}.	10^{-9}.	10^{-10}.
Cucumber 3 . . .	+ +	+ +	+ +	— —
,, 3 . . .	+ +	+ +	+ +	+ —
,, 3 . . .	+ +	+ +	+ —	— —
,, 4 . . .	+ +	+ +	+ —	+ —
,, 4 . . .	+ +	+ +	+ +	— —
,, 4 . . .	+ +	+ +	— —	— —

The dilution is given in grammes of protein per c.c. of inoculum and two plants were inoculated with 1 c.c. each at every dilution. The + sign indicates systemic infection and the — sign indicates that the plant remained healthy.

Tobacco (var. White Burley), tomato, *Nicotiana glutinosa,* and Golden Cluster beans, which are all susceptible to tobacco mosaic virus at high dilutions, did not become infected when inoculated with solutions containing as much as 1 p.c. of the nucleoproteins isolated from infected cucumber plants.

The purified preparations of cucumber viruses 3 and 4 are antigenic, and antisera precipitating at a dilution of 1 in 1000 were produced by giving rabbits a single intravenous injection of 5 mg. With these antisera the serological titres of the virus preparations were determined by the technique previously described, 1 c.c. of virus solution at various dilutions being mixed with 1 c.c. of antiserum at a constant dilution. The highly purified, liquid crystalline, preparations of cucumber viruses 3 and 4 have given precipitation end points of from $\frac{1}{6 \times 10^6}$ to $\frac{1}{8 \times 10^6}$. The activity is, therefore, very similar to that of purified tobacco mosaic virus, and the serological titres are of the same order as those obtained with other highly purified antigens.

The serological titre of the material as normally prepared gives an accurate index of its infectivity, but certain treatments, e. g. with nitrous acid or exposure to X-rays, destroy infectivity without affecting the serological reactions. Preparations of cucumber viruses 3 and 4, rendered non-infective by such methods, still show anisotropy of flow and form liquid crystalline solutions, and when precipitated with acid or ammonium sulphate form para-crystals indistinguishable from those of fully active virus.

The infectivity of our purified virus preparations is not sufficiently great for us to state that they contain only virus particles, and that the nucleoproteins are therefore necessarily the viruses. The high serological titres and data from centrifuging and X-ray experiments suggest that our material is not grossly contaminated, but there remains the possibility that the nucleoproteins

we have isolated are antigens peculiar to the infected plants, and that they are " contaminated " with small amounts of virus responsible for the infections. If this is so, then we must assume that in addition to the viruses themselves multiplying in the infected plants they also produce (or cause to be produced) the nucleoproteins, for these have not been isolated from healthy plants. We have no evidence that infectivity can in any way be dissociated from the nucleoprotein : infectivity is closely correlated with the amount of protein, and loss of infectivity is accompanied by changes in the protein, and denaturation of the protein by heat, acid or alkali is accompanied by loss of infectivity.

At the present stage of the work it seems most reasonable to assume that the nucleoproteins are the viruses, but to remember that the point is not proved, and the possibility still exists that the nucleoproteins are disease products to which the viruses are firmly attached.

Cross-precipitation tests with cucumber virus 3 as antigen and tobacco mosaic virus antiserum, and *vice versa*, have shown that the two viruses are serologically related, for both antigens precipitate with both antisera. The serological titres given by preparations of either virus are practically independent of the antiserum used, but the range of antigen dilution over which precipitation takes place varies greatly with different antisera. When the cucumber viruses are titrated against a constant amount of the antiserum to any of the three strains of tobacco mosaic virus which we have used, there is a central zone of precipitation with large zones of non-precipitation in the antigen excess region (see Table II). When any of the three tobacco mosaic viruses are titrated against a constant amount of antiserum to cucumber virus 3 at the same dilution there are similar zones of non-precipitation in the antigen excess region. When cucumber viruses 3 and 4 are titrated against cucumber virus 3 antiserum, or when any of the three strains of tobacco mosaic virus are titrated against either their own or each other's antiserum, the zones of non-precipitation in the antigen excess region occur only where the antigen is very much more concentrated, and the zones are therefore smaller. The results of an experiment in which tobacco mosaic virus and cucumber virus 3 were titrated against the four antisera are shown in Table II. It will be seen that there are differences in the range of precipitation of the antisera to the individual strains of tobacco mosaic, but these are extremely small in comparison with the differences between the tobacco and the cucumber viruses.

Many virus workers have shown that the individual strains of plant viruses are serologically related, and at the present time it seems that all viruses which have the power of immunizing plants against one another are serologically related. Only Chester (1936) has shown that the strains are not serologically identical. Chester, using clarified infective sap as his antigens, found that he could absorb tobacco mosaic virus antiserum with Aucuba mosaic virus and then get no precipitate with the latter, but still get a precipitate with tobacco mosaic virus. Similarly, if Aucuba mosaic virus antiserum was fully absorbed with tobacco mosaic virus it would still precipitate with Aucuba mosaic virus. Chester also found that some of the strains isolated by Jensen (1933) were serologically identical with tobacco mosaic virus and others with Aucuba

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TABLE II.—*Precipitation of Tobacco Mosaic Virus and Cucumber Virus 3 with Different Antisera.*

1. Tobacco Mosaic Virus as Antigen.

Antiserum	Time	Dilution of antigen (1/1 = 1 mg. per c.c.).					
		1/1.	1/4.	1/16.	1/64.	1/256.	1/1024.
Tobacco mosaic virus	2 min.	+++	+++	+	+++	++	+
	2 hr.	+++	+++	+++	+++	+++	++
	24 ,,	+++	+++	+++	+++	+++	+++
Aucuba mosaic virus	2 min.	+++	+++	+	+++	++	+
	2 hr.	+++	+++	+++	+++	+++	++
	24 ,,	—	—	+	—	—	—
Enation mosaic virus	2 min.	—	+++	+++	+++	++	+++
	2 hr.	—	+++	+++	+++	+++	+++
	24 ,,	—	—	—	+	+	+
Cucumber virus 3	2 ,,	—	—	—	+	++	+++
	24 ,,	—	—	—	—	++	++

2. Cucumber Virus 3 as Antigen.

Antiserum	Time	Dilution of antigen (1/1 = 1 mg. per c.c.).					
		1/1.	1/4.	1/16.	1/64.	1/256.	1/1024.
Tobacco mosaic virus	1 hr.	—	—	—	+	++	—
	24 ,,	—	—	—	++	+++	++
Aucuba mosaic virus	2 ,,	—	—	—	—	+	—
	24 ,,	—	—	—	—	++	++
Enation mosaic virus	2 ,,	—	—	+	—	—	—
	24 ,,	+++	+	+	—	—	—
Cucumber virus 3	2 min.	+++	+++	—	—	—	—
	2 hr.	+++	+++	+++	+++	++	++
	24 ,,	+++	+++	+++	+++	+++	+++

In all tests the antiserum was used at a dilution of 1/50. 1 c.c. of antiserum was added to tubes containing 1 c.c. of antigen at given dilutions, and the tubes were immediately placed in a water-bath at 50° C.

+ signs indicate the degree of precipitation.

mosaic virus. Chester does not seem to have considered the possibility of quantitative antigenic differences and his tests seem to have been entirely qualitative ; after the sera had been absorbed, tests for further precipitation were made at only one antigen or antiserum dilution. The tests in which no precipitation was obtained do not therefore necessarily indicate that the serum was completely absorbed, as Chester states, for they may have been made in a region of antigen excess which inhibited precipitation, as shown in Table II. Using purified virus preparations we have made cross-absorption experiments between our virus strains and their antisera, and we have found differences of the same type as those described by Chester. In these tests, as in the straightforward precipitation tests, much greater differences were found between the cucumber and the tobacco viruses than were found between the individual strains of the tobacco viruses. In each test a preliminary experiment was made to determine the optimal combining proportions of the antigen (virus) used for the absorption and the antiserum to be absorbed. The optimal combining proportions were determined by adding 1 c.c. of antigen at different concentrations to each of a series of tubes containing 1 c.c. of serum at a constant dilution. The tubes were immediately placed in a water-bath at 50° C. and the proportions of antigen and antiserum in that tube which first showed a precipitate were taken as optimal (Topley and Wilson, 1936, p. 144). Antigen and antiserum were then mixed with the antigen in slight excess of its optimal value ; the mixture was then incubated for 2 hours at 50° C., placed in the ice-box overnight and then centrifuged. The supernatant fluid was then tested at a constant dilution against the antigen used for absorption at a number of different antigen dilutions. If there was no further precipitation it was then tested against the other virus strains, but if it still precipitated a second absorption was made. The sera, fully absorbed with one virus strain, were then tested for their ability to precipitate with the different virus strains, the tests being made with antiserum at a constant dilution and with antigen varying over a wide range of dilutions.

The amount of virus required to absorb an antiserum varied greatly with the different antigens and antisera : it was greatest when antigens were used to absorb their homologous antisera, somewhat less when the strains of tobacco mosaic virus were used to absorb each other's sera, and very much less when cucumber virus 3 was used to absorb tobacco mosaic virus antiserum or tobacco mosaic virus to absorb cucumber virus 3 antiserum. When tobacco mosaic virus antiserum was absorbed with cucumber virus 3 the precipitation of the serum with tobacco mosaic virus was only slightly affected, and the optimum precipitation point was only slightly shifted. Similarly, when cucumber virus 3 antiserum was completely absorbed with any of the three tobacco mosaic virus strains, its precipitation with cucumber virus 3 was only slightly affected. On the other hand, when antiserum to one strain of tobacco mosaic virus was fully absorbed with another strain its precipitation with the strain used for immunization was greatly affected, and the optimum precipitation point showed a large shift.

In Table III are shown the summarized results of several experiments in which the various antisera were absorbed with different virus strains and then

TABLE III.—*Summarized Results of Cross-absorption Experiments.*

Precipitation tests with absorbed sera and antigens.

Antiserum absorbed.	Antigen used for absorption.	Antigen.				
		Tobacco mosaic virus.	Aucuba mosaic virus.	Enation mosaic virus.	Cucumber virus 3.	Cucumber virus 4.
Tobacco mosaic virus	T.M.V.	—	—	—	·	·
	A.M.V.	++	++	++	·	·
	E.M.V.	++++	++++	+++	·	·
	C.V. 3	++++	++++	+++	·	·
	C.V. 4	++	++	++	·	·
Aucuba mosaic virus	T.M.V.	++	++	++	·	·
	A.M.V.	++++	++++	+++	·	·
	E.M.V.	++++	++++ +	+++	·	·
	C.V. 3	++++	++++	+++	·	·
	C.V. 4	++	++	++	·	·
Enation mosaic virus	T.M.V.	—	—	—	·	·
	A.M.V.	—	—	—	·	·
	E.M.V.	++++	++++	+++	·	·
	C.V. 3	++	++	++	·	·
	C.V. 4	++	++	++	·	·
Cucumber virus 3	T.M.V.	—	—	—	+++	+++
	A.M.V.	—	—	—	+++	+++
	E.M.V.	—	—	—	+++	+++
	C.V. 3	·	·	·	·	·

— indicates that there is no precipitation.
+ signs indicate the degree of precipitation at the optimum.　For description of method see text.

tested for their precipitability with other virus strains. From these it is apparent that tobacco mosaic antiserum absorbed with Aucuba mosaic virus contains no residual precipitating antibodies, while when absorbed with Enation mosaic virus it still precipitates with Aucuba mosaic and tobacco mosaic viruses ; Aucuba mosaic virus antiserum after absorption with Enation mosaic virus still precipitates with both tobacco mosaic and Aucuba mosaic viruses, and after absorption with tobacco mosaic virus it still precipitates with Enation mosaic and Aucuba mosaic viruses ; Enation mosaic virus antiserum after absorption with Aucuba mosaic virus will still give a slight precipitate with Enation mosaic virus but none with tobacco mosaic virus, and after absorption with tobacco mosaic virus it precipitates with both Aucuba mosaic and Enation mosaic viruses.

The results show that in addition to an antigenic fraction common to the three strains of tobacco mosaic virus examined, tobacco mosaic virus and Aucuba mosaic virus contain a fraction not present in Enation mosaic virus, Aucuba mosaic virus and Enation mosaic virus contain a fraction not present in tobacco mosaic virus, and Enation mosaic virus contains a fraction not present in Aucuba mosaic virus. If each fraction distinguished be represented by a letter, then the simplest formulæ for the three strains which can adequately explain the results are :

Tobacco mosaic virus A B.
Aucuba mosaic virus A B C.
Enation mosaic virus A — C D.

It is to be expected that the use of a larger number of strains in the cross-absorption experiments would have shown further differences between the three strains, and it is possible that each of the symbols in the formulæ represent groups of antigens rather than single antigens. If we assume that each of the components has an antigenicity of the same order as the others it would appear that the common fraction A is predominant ; for removal of the antibodies to A greatly reduces the power of an antiserum to precipitate. There is, however, no definite evidence on the relative quantities of the different antigens present in these strains.

When tobacco mosaic virus antiserum is absorbed with either Enation mosaic or Aucuba mosaic virus it loses its power of precipitating with cucumber virus 3. The antigens which tobacco mosaic virus shares with cucumber virus 3 therefore must be contained in the common fraction A. The serological relationship between tobacco mosaic virus and cucumber virus 3 is best explained by postulating that, in addition to the antigens specific to each, the two viruses have two common antigens, and that while the total of the two antigens in each is of the same order, tobacco mosaic virus contains a preponderance of one and cucumber virus 3 a preponderance of the other. If we call the common antigens X and Y, then tobacco mosaic virus might be indicated as (NX nY) and cucumber virus 3 (nX NY). The antibody response in the rabbit will probably not be directly proportional to the amount of each antigen present, but the quantitative difference between the two components of the antiserum will be smaller than that between the two components of

the antigen ; for, during the immunization of the rabbit, the major antibody response will reach a maximum while the minor antibody concentration in the serum is still increasing. If we take the purely arbitrary values of 80 for N and 1 for n, then tobacco mosaic antiserum might have the structure 80 anti-X and 10 anti-Y. Similarly cucumber virus 3 would be 1X and 80 Y while its antiserum would be 10 anti-X and 80 anti-Y. If these units are taken as representing optimal combining amounts, i. e. if 1 unit of X combines with 1 unit of anti-X and 1 unit of Y with 1 of anti-Y, and if a precipitation test with constant antiserum is considered using both homologous and heterologous antigen, then a result of the type shown in Table IV would be expected if we assume that inhibition in the region of antigen excess takes place as in the experiments that we have already described.

TABLE IV.—*Antiserum (Tobacco Mosaic Virus).*

Anti-X	80	80	80	80	80	80	80
Anti-Y	10	10	10	10	10	10	10
Homologous antigen X	320	160	80	40	20	10	5
concentration Y (T.M.V.)	4	2	1	..	..	..	..
X-effect	+++	++++	++++	++++	+++	++	+
Y-effect	+	—	—	—	—	—	—
Combined effect	++++	++++	++++	++++	+++	++	+
Heterologous antigen X (C.V. 3)	4	2	1	..	..	..	..
Y	320	160	80	40	20	10	5
X-effect	—	—	—	—	—	—	—
Y-effect	—	—	—	+	++	++	+
Combined	—	—	—	+	++	++	+

It will be seen on reference to Table II that results of this type are obtained when either tobacco mosaic virus or cucumber virus 3 are titrated against their homologous and heterologous antisera, the antisera being used at a constant dilution, and that the large zones of non-precipitation obtained with the heterologous antisera can be explained on the basis that the precipitates are produced only by the minor antibody and the major antigen. An antigenic relationship of this type also explains the results of the cross-absorption experiments, for it is obvious that absorbing at the constant serum optimum with the heterologous antigen will remove all the minor antibody but only slightly reduce the major antibody, and the precipitation of the serum with its homologous antigen will be but little affected.

Although the antisera to the different viruses are demonstrably different in their precipitating antibody content, no differences have been found in their virus-neutralizing antibodies. When antisera to cucumber virus 3 or to any of the three strains of tobacco mosaic virus were mixed *in vitro* with any of the three strains of tobacco mosaic virus, they all proved equally effective in reducing the number of local lesions produced in *N. glutinosa*. The neutralizing effect is approximately proportional to the concentration of the antiserum and no " zone phenomena " have been noted.

VIRUS-CLUPEIN PRECIPITATES.

We have already shown (Bawden and Pirie, 1937b) that a paracrystalline precipitate, closely resembling that obtained with acid or ammonium sulphate, develops when neutral solutions of clupein and tobacco mosaic virus are mixed, and that the solubility of the precipitate in salt solution varies with the different virus strains. This phenomenon has now been investigated more fully, and certain similarities and differences in the behaviour of the three tobacco and two cucumber viruses have been determined. The precipitation appears to occur immediately the virus and clupein are mixed ; it is greatly affected by changes in pH value and salt concentration, but is unaffected by small variations in temperature.

In the experiments described the amount of purified virus used was constant at 1 mg., while the other components of the system were varied and the final volume of the mixtures was 3·5 c.c. Readings of the extent of the precipitation were made by measuring the opacity of the suspensions with a photoelectric cell. When mixed with neutralized solutions of clupein sulphate (B.D.H.) none of the viruses gave a perceptible precipitate with 0·006 mg., and all gave maximum opacity with 0·05 mg. On the basis of their behaviour with intermediate quantities of clupein sulphate it was possible to divide the viruses into two groups ; for tobacco mosaic and Enation mosaic viruses are more easily precipitable than Aucuba mosaic virus or either of the cucumber viruses : for example, with 0·04 mg. both tobacco mosaic and Enation mosaic viruses developed rather more than 50 p.c. of the maximum opacity, whereas the other three developed only 10 p.c.

Much more striking differences are obtained by varying the pH values and the salt contents of the mixtures, and these effects lead to a much better defined division of the viruses into two groups. When a system containing 0·06 mg. of clupein sulphate and 1 mg. of virus in 3·5 c.c. is studied, it is found that the precipitates with Enation mosaic and tobacco mosaic viruses are less soluble in the presence of salt than the precipitates with the other three viruses. For example, at pH 5·5 a suspension of the clupein compounds of Enation mosaic or tobacco mosaic virus in $M/60$ phthalate buffer has only half the opacity of a similar suspension in water, whereas with the other three viruses it is only necessary to raise the salt concentration to $M/200$ at this pH to produce this reduction in opacity. On either side of pH 5·5 the precipitates with all 5 viruses become less soluble in salt, and approximately twice the concentration is necessary to reduce the opacity to half at pH 5 or 6 ; thereafter the solubility rises again. The exact behaviour depends to a slight extent on the clupein-virus ratio, the strain of virus used and the particular salt ; it is therefore unprofitable to attempt at present to give any great precision to a description of the system. It is clear, however, that under similar conditions the solubility minima have nearly the same pH values for all five viruses.

How far these results are generally applicable to viruses is unknown, but purified preparations of potato virus " X ", which are also liquid crystalline, give with clupein sulphate an amorphous precipitate which is presumably

analogous to the para-crystalline precipitates described here. In interpreting histological appearances of virus-infected plants it is important that the existence of these complexes should be realized, for they are insoluble under conditions which may exist in the interior of cells, and similar virus precipitates are formed with some histones and protamines other than clupein.

It is well known that plants infected with strains of tobacco mosaic virus contain intracellular inclusions of two types. The one consists of rounded vacuolate bodies (X-bodies) and the other of flat plates. Iwanowski (1903) showed that the latter become striated when made acid, and Goldstein (1926) confirmed this, and stated that when acidified they seemed to be made up of distinct rods or needle crystals. Beale (1937) has pointed out that the needles obtained by acidifying these plates closely resemble the " crystals " described by Stanley (1936) in his acidified tobacco mosaic virus preparations, and suggests that the plates are the source of the virus. Examined between crossed Nicol prisms the plates are seen to be birefringent when viewed edgeways but not when viewed flat. As many of the plates are definitely hexagonal this suggests that they may be true hexagonal crystals, for these are birefringent only when viewed along the transverse axes. Highly purified preparations of all five viruses give birefringent solutions, jellies and paracrystals, but no true crystals comparable to those seen in plants infected with strains of tobacco mosaic virus have yet been prepared. If, as the evidence suggests, the hexagonal crystals are depositions containing virus, there are several possible explanations for the different behaviour in the plant and after isolation. In the plant a process of slow crystallization which has not been simulated *in vitro* may go on ; or the plates may be composed of a virus-host complex similar to the precipitates we have described with clupein ; or the different crystalline states may have their origin in the different physical states of the virus before and after isolation. We have previously produced evidence indicating that during the process of purification the virus undergoes an irreversible aggregation, and it is possible that small units as they occur in the plant can arrange themselves in true crystals, whereas the larger aggregates in the isolated virus preparations cannot. The X-bodies are apparently quite different structures from these plates, for they are not birefringent, and Beale (1937) has shown that they are unaffected by acid.

In cucumber plants infected with viruses 3 and 4 no intracellular inclusions have been seen, and if they occur it must be much more rarely than in solanaceous plants infected with the strains of tobacco mosaic virus. The source of the virus therefore cannot lie entirely in the inclusions, as Beale suggests, but the absence of crystals from infected cucumbers might lend support to the idea that their production is a function of the concentration of virus ; for, as we have stated above, the yield of cucumber viruses per volume of expressed sap is much less than that of the tobacco mosaic viruses. Other factors, however, might equally well explain the absence of inclusions : for example, the sap of cucumbers is very much more alkaline than is that of solanaceous plants, and this would increase the solubility of either the viruses or of virus complexes of the type we have described.

DISCUSSION.

It has been realized for some years that certain plant viruses occur in numerous strains : in general the strains possess similar host ranges and properties, but are differentiated because they cause different symptoms. A considerable amount of circumstantial evidence has also been accumulated, indicating that strains are continuously arising by a process analogous to mutation. Various workers have shown that recognized virus strains are closely related serologically, and that they have the power of immunizing plants against one another. A relationship of the type that exists between the tobacco mosaic viruses and cucumber viruses 3 and 4 does not seem to have been described before. Cucumber viruses 3 and 4 were recognized to be related strains, but because of their different host range they had not previously been thought to be related to tobacco mosaic virus. From our results it is apparent that the five viruses studied fall into one main group ; the analytical figures for all are so similar that they afford no differentiation, and the differences in the physical and chemical properties yet found are on the whole trivial. They are sufficient, however, to show that the nucleoproteins isolated from plants infected with strains differentiated on phytopathological grounds are different proteins. They also show that the greater the differences in host range and symptoms caused, the greater the differences that can be detected in the properties of the isolated viruses, and suggest the advisability of differentiating and grouping viruses by other than usual phytopathological methods. Of these, the serological technique and X-ray analysis would appear to be most useful. Straight precipitation tests are sufficient to distinguish between the tobacco mosaic viruses and the cucumber viruses, but to distinguish between the individual strains of tobacco mosaic virus the more sensitive cross-absorption test must be used. The X-ray measurements (Bernal and Fankuchen, 1937) show differences of the same order : all five viruses pack in the same manner, indicating that they are of the same general shape, but measurements of the main spacings are sufficient to distinguish between the tobacco mosaic viruses and the cucumber viruses, the latter having a smaller cross-section. The three strains of tobacco mosaic virus all give the same main spacings, but a consideration of the relative intensities of all the lines on the X-ray plate separates each strain with certainty from the others.

The relationship of cucumber viruses 3 and 4 to the tobacco mosaic viruses is difficult to define. It was suggested (Bawden, 1934) that, in discussing the potato virus " X " group, relationships are found analogous to those indicated by genera, species and varieties. If this view be adopted here then all five viruses examined could be regarded as belonging to one genus, cucumber viruses 3 and 4 as varieties of one species and the three strains of tobacco mosaic virus as varieties of a second species of the same genus.

Most plant viruses are not serologically related to tobacco mosaic virus and possess quite different properties *in vitro*. The fact that the five viruses described form such an uniform group gives us no reason to imagine that all other plant viruses are necessarily similar in their chemical properties.

SUMMARY.

Methods are described for the isolation of nucleoproteins from cucumber plants infected with cucumber viruses 3 and 4. These have not been isolated from uninfected plants, and all the available evidence indicates that they are the viruses themselves. Infections were obtained with 10^{-10} g., and specific precipitates with antiserum with $1/8 \times 10^{-6}$ g. Concentrated solutions are spontaneously birefringent and dilute solutions show anisotropy of flow : when sedimented by high-speed centrifugation they form birefringent jellies, and when precipitated with acid or ammonium sulphate they form needle-shaped para-crystals. Although these viruses have a distinct host range from tobacco mosaic virus, the purified preparations have similar chemical composi-tions and many properties in common with purified preparations of strains of tobacco mosaic virus ; they differ from tobacco mosaic virus, however, more widely than the recognized strains of tobacco mosaic virus differ from each other. The cucumber viruses and the tobacco mosaic viruses have common antigens : the results of cross-absorption experiments between the various viruses and their antisera are described, and provisional antigenic formulæ suggested. Possible methods of relating and distinguishing between viruses and the relationship between the cucumber and tobacco viruses are discussed.

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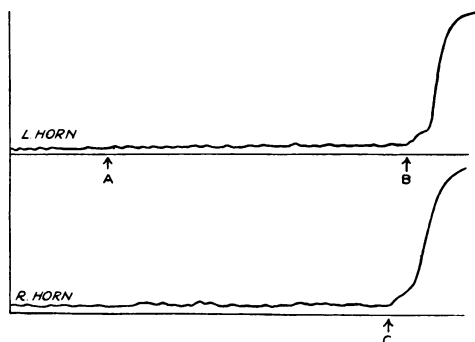
A NOTE ON ANAPHYLAXIS WITH TOBACCO MOSAIC VIRUS PREPARATIONS.

Chester (1936) showed that normal tobacco protein is strongly anaphy-lactogenic but that tobacco mosaic virus is not, for guinea-pigs sensitized with sap from infected plants could be desensitized completely with the sap

from healthy plants of the same species. Chester tested several preparations
of tobacco mosaic virus prepared by Stanley, and although he found that they
were not active anaphylactogens, yet they all reacted with guinea-pigs sensitized
with healthy tobacco sap. Because of this Chester stated that the prepara-
tions of tobacco mosaic virus then available (1936) contained demonstrable
amounts of normal plant proteins.

Dr. K. S. Chester has kindly tested one of our highly purified spontaneously
birefringent solutions of tobacco mosaic virus which had been subjected in
the course of the preparation to treatment with trypsin (Bawden and
Pirie, 1937). This is the only preparation in which he has been unable to
demonstrate the presence of normal protein by the anaphylactic test.

Thirteen pigs were injected by Dr. Chester with doses varying from
0·1 to 10·0 mg. of our preparation. The uterine horns from these pigs gave no

Kymograph tracing with the two horns of the uterus of a guinea-pig sensitized with 1 mg. of
normal tobacco protein 19 days before. At A, 10 mg. of our preparation was added, at
B 1·5 mg. of normal tobacco protein, and at C, 5 mg. of a preparation made by Stanley's
technique.

reaction with normal tobacco protein or with tobacco mosaic virus prepara-
tions made by us or in the U.S.A. by Stanley's technique.

Six guinea-pigs sensitized with 1 mg. of normal tobacco protein reacted
strongly both with tobacco protein (1·5 mg.) and with tobacco mosaic virus
(5 mg.) preparations made by Stanley's methods, involving repeated " re-
crystallizations " and ultracentrifuging twice, but they gave no reaction with
10 mg. of our product. The results of one of these tests are shown in the
figure.

We have previously suggested that the needles precipitated from solutions
of tobacco mosaic virus by means of acid or ammonium sulphate are not true
crystals, and that there is no reason to assume that preparations are necessarily
pure because their properties are unaffected by repeated " recrystallization ".
Dr. Chester's results show quite clearly that incubation with trypsin readily
effects a fractionation that cannot always be obtained by precipitation methods
or by centrifugation.

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