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The inhibition, inactivation and precipitation of tobacco mosaic virus nucleic acid by components of leaf extracts

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Unless calcium is removed from leaf extracts containing tobacco mosaic virus nucleic acid, infectivity measurements are invalid. In some circumstances, calcium, nicotine and spermine can prevent the movement of nucleic acid into the water phase when extracts are made in the presence of excess phenol. The concentrations needed for this effect are greater than those usually found in leaf extracts, but it is possible that association of the virus with these substances produces local concentrations large enough to influence the infectivity of extracts.

Introduction

During the early stages of the work described in the preceding paper (Bawden & Pirie 1972), it seemed possible that differences in the infectivity of extracts, made in the presence of phenol, from tobacco plants infected with tobacco mosaic virus (TMV), were the consequence of inactivation by diffusible components of the leaf. When it became clear that these differences were mainly caused by differences in the fixation of RNA to leaf fragments, work on inactivations in solution became less relevant. Nevertheless, in some circumstances, infective RNA from TMV (TMV:RNA) is inhibited by leaf components – that is to say, infectivity is restored when the inhibitor is removed. Other components remove RNA from aqueous solution, or cause irreversible inactivation.

There are now many publications on the infectivity of extracts, made in the presence of phenol, from plants infected with various viruses, from which the phenol was extracted with ether so that it would not damage the test-plants. Ether-treated extracts from plants infected with TMV are less infective than dialysed extracts and, unlike dialysed extracts, they sometimes produce more lesions when diluted than when used concentrated. Also, second and third extracts, in which it is reasonable to suppose that the concentration of TMV:RNA and other soluble components will be less than in first extracts, are sometimes more infective than first extracts. The infectivity of extracts freed from phenol by ether extraction is increased if they are then dialysed, but they never attain the infectivity of extracts dialysed immediately. It seems therefore that there is both inhibition and inactivation in these extracts and that an examination of the influence of various leaf components on the infectivity of fluids containing TMV:RNA is essential before the validity of infectivity measurements can be assessed.

† Sir Frederick Bawden, Treas.R.S. died on 8 February 1972 while this paper was in final preparation.

Materials and methods

The methods described in the preceding paper (Bawden & Pirie 1972) were used to measure infectivity, prepare TMV, and make leaf extracts.

Preparation of TMV:RNA

An aqueous solution containing 30 g of carefully purified TMV (Pirie 1956) and 3 g of trisodium citrate per litre is shaken vigorously for a few seconds with half its weight of phenol, centrifuged, and the supernatant fluid shaken again with phenol. This time the two layers usually separate without centrifuging. The aqueous layer, after dialysis, contains about 1.5 g/l TMV:RNA, but the value depends on the tension imposed on the content of the dialysis tube.

Assay of TMV:RNA inactivators

Except during summer, when more concentrated inoculum is needed, 10 μg TMV:RNA in 2 ml usually gives a satisfactory number of necrotic lesions on eight half leaves of the tobacco variety Xanthi, n.c. A stock inoculum containing 0.5 g/l of TMV:RNA in water was prepared and divided into several batches, which were kept at −20 °C and thawed just before use. Several cycles of freezing and thawing cause no apparent loss of infectivity. Leaf fractions, or other solutions to be tested for their ability to inactivate, were usually made up to 0.38 ml in 3 ml tubes cooled in ice, then 0.02 ml of the stock TMV:RNA was added; after 3 h at 0 °C, the mixtures were diluted by adding 1.6 ml of 5 mM K_2HPO_4, and kept at 0 °C until they were inoculated to plants. The interval between dilution and inoculation seldom exceeded 1 h, but this is not critical because dilution curbs all actions. The action of leaf ribonuclease (RNase), in those experiments in which it was added as an inactivating agent, is curbed by the alkaline pH established by phosphate.

Results

Calcium and other cations

The infectivity of intact TMV is not affected by Ca^{2+} in the inoculum at concentrations within the physiological range, but multiplication of TMV in tobacco plants grown in sand culture is reported to be hastened in plants watered with a nutrient solution 1 mM with respect to Ca^{2+}; 9 mM Ca^{2+} enhanced plant growth but slowed virus multiplication (Varma & Verma 1963). Unfortunately the concentration of Ca within the plant was not measured, but a recent survey (Burström 1968) suggests that the concentration within the plant would increase with increasing concentration in the nutrient solution. Burström also quotes evidence that membrane permeability is affected by changes in Ca content. Some physiologically controlled differences in the apparent susceptibility of plants to infection with TMV may therefore be the result of differences in the readiness with which

the infective agent spreads – especially if the agent is TMV:RNA rather than TMV.

Calcium is held in the leaf in many different ways and, as in other tissues (cf. Legato & Langer 1969), there are regions of local concentration. The concentration in an extract may therefore depend on the way the extract was made, and the proportion of the Ca that is ionic will depend on the chelating agents present. If all the Ca that is extracted by boiling 2% acetic acid from the leaves of the plants we use were ionic and in solution in all the water of the leaf, that solution would be 100 to 200 mM. The larger values are given by the lower two or three leaves. However, not all the Ca is in solution, not all the water is available for its solution, and our process of extraction involves a threefold dilution. The observation that extracts made at pH 8 without phosphate contain 10 to 20 mM of Ca precipitable by oxalate is therefore reasonable. Extracts made in the presence of phosphate contain about 4 mM. The effects of Ca^{2+} in the inoculum in the concentration range 4 to 20 mM is therefore relevant in interpreting results with undialysed extracts.

TABLE 1. THE EFFECT OF DIFFERENT CONCENTRATIONS OF CALCIUM ON THE NUMBER OF LESIONS PRODUCED ON TEST-PLANTS OF DIFFERING AGE

Each pair of columns gives the numbers of lesions on opposite halves of the leaves of one plant on to which a 5 mg/l solution of TMV:RNA, containing the stated amount of calcium acetate, had been rubbed.

concentration of Ca^{2+} in inoculum/mM	young plants				totals
0	13 30	12 36	40 8	60 40	239
5	30 22	11 9	40 50	34 12	208
10	0 0	1 4	16 6	17 16	66
20	0 0	0 1	2 2	13 6	24
	medium plants				
0	2 9	9 2	30 30	10 22	114
5	7 9	1 2	4 20	2 1	46
10	3 4	4 2	1 0	1 1	16
20	0 0	0 0	0 0	1 1	2
	old plants				
0	0 2	8 1	4 1	7 5	28
5	0 1	1 1	1 0	1 7	12
10	3 0	3 2	0 1	0 3	12
20	0 0	0 0	0 0	0 0	0

Table 1 shows the extent of this inhibition on test plants of three ages. The number of lesions on each half-leaf is given to show how variable results are when TMV:RNA is the inoculum. Each of the four pairs of columns give the number of lesions on half-leaves on the same plant and the great susceptibility to infection in the presence of Ca^{2+} of one of the young plants (the last pair of columns) is noteworthy.

Dialysis of solutions containing little besides TMV:RNA and diluted to the extent usual for an inoculum, tends to give variable results; infectivity is seldom completely restored, but much of it always is. Variability is probably the result of both adsorption and oxidation because it is minimized by dialysing against 5 mM K_2HPO_4 rather than water, and by excluding air. The infectivity of a dilute solution of TMV:RNA, in a shallow vessel containing a piece of washed 'Cellophane' or filter paper, diminishes in a few hours at 0 °C.

The inhibitory effect of Ca^{2+} suggested that some differences in the susceptibility of different batches of leaves might be caused by Ca^{2+} at or near the leaf surface. This would explain the increased number of lesions often observed when the inoculum contains phosphate and some other substances. However, EDTA in the inoculum caused, if anything, a slight decrease in the number of lesions. Photochemical destruction of EDTA on the leaf surface was probably not the reason for the lack of effect because results were similar in the light and dark.

With different batches of test plants, between 4 and 10 mM Ca^{2+} is needed to get unequivocal diminution in the number of lesions caused by an inoculum. The necessary concentration is consistently smaller at pH 8 than 6. This contrasts with the effect of pH on the infectivity of TMV:RNA without Ca^{2+}; then the more alkaline solutions produce more lesions. The diminished infectivity of alkaline solutions of TMV:RNA containing Ca^{2+} is probably caused by aggregation or incipient precipitation; an inoculum with undiminished TMV:RNA content would then contain fewer separate particles. At the TMV:RNA dilution used in an inoculum, and with 10 mM Ca^{2+}, precipitation is not visible but it can be demonstrated by centrifuging, at 10000 rev/min, dilute mixtures that have been made up for a day. Behaviour is erratic after shorter exposure. In more concentrated mixtures, precipitation is obvious. A 1 g/l solution of TMV:RNA in 10 mM pH 8 tris(hydroxymethyl)methylamine precipitates immediately at 0 °C with 100 mM Ca^{2+}, in an hour with 30 mM and 24 h with 10 mM.

The presence of various salts prevents precipitation, and it is obviously not complete in the presence of the usual components of tobacco sap or sap would not be infective after treatment with phenol. Undiluted sap from infected plants contains 2 to 3 g TMV, i.e. 100 to 150 mg of TMV:RNA, per litre. The extracts used in the preceding paper (Bawden & Pirie 1972) were usually diluted three- or fourfold. After dialysis, they precipitate in a few hours with 100 mM Ca^{2+} and the precipitate is fully infective when, redissolved in citrate, provided the Ca^{2+} solution used for precipitation is carefully purified. The presence of traces of iron probably explains the inactivation we reported (Bawden & Pirie 1959); that paper also commented on the sensitivity of TMV:RNA to dilute solutions of iron salts. At that time, we confused two phenomena: reversible inhibition by Ca^{2+}, and irreversible inactivation by contaminants in the material used.

The conclusion from these observations is that the number of lesions produced by a leaf extract will have little bearing on the amount of infective TMV:RNA in

it unless it is largely freed from Ca^{2+}, by dialysis or in other ways, before assay.

The preceding paper describes the role of Ca^{2+} in promoting fixation of TMV:RNA on to leaf fibre. Although other components of leaf extracts keep TMV:RNA from precipitating completely with Ca^{2+} at the concentrations usually present, their effect is counteracted by adding more Ca^{2+}. Extracts made from infected leaves at pH 7.6 in the presence of 28 mM phosphate and excess phenol, give hundreds of lesions after dialysis, whereas they give few if any when 50 mM Ca^{2+} is included in the mixture. With less Ca^{2+}, results are less clear. Extracts made without phosphate behave in the same way, so the effect is not simply adsorption on calcium phosphate. There is less complete removal of infectivity from the extract if it is just saturated with phenol without a phenol phase being present.

The infectiveness of ultimately dialysed solutions containing 10 mg/l purified TMV:RNA in 10 mM piperazine-*NN'*-bis-2-ethane-sulphonic acid at pH 7.6, is not altered by the presence of 10 mM Ca^{2+} or excess phenol separately. When both are added and the dialysed aqueous layer is dialysed immediately, its infectivity is diminished; when phenol is added after 24 h exposure to the Ca^{2+}, loss of infectivity is nearly complete. When these solutions are distilled to dryness to remove the trace of phenol that remains after dialysis, light absorption at 260 nm (the buffer does not absorb at 260 nm) by the redissolved residue parallels infectivity closely. This shows that TMV:RNA is removed from the aqueous layer rather than inactivated in it. Recovery from the phenol layer has never been complete, whether measured by light absorption at 260 nm or by infectivity.

Mg^{2+} is less inhibitory than Ca^{2+} and there is less of it in the leaf; it is therefore less likely to cause confusion. Mn, at physiological concentrations, has little effect when added to the inoculum. Although the concentration of Zn in leaf extracts is so small that it is not likely to affect the behaviour of RNA, Zn^{2+} is such a powerful inhibitor of some RNases (Holden & Pirie 1955) that we tried to control residual RNase activity in early preparations of TMV:RNA by adding it to TMV before disruption with phenol. It proved even more effective than Ca^{2+} in removing TMV:RNA from the aqueous phase.

Various other salts, e.g. potassium chloride and ammonium sulphate, which may occur in preparations made during the preliminary fractionation of leaf extracts, are inhibitory – but only at concentrations near the limit at which there is osmotic damage to the leaf.

Nicotine

In the pH range 6 to 8, 24 h exposure to nicotine at 20 g/l destroys the infectivity of TMV:RNA. Destruction is incomplete with more dilute nicotine, shorter periods of exposure, or in the presence of some salts. There is little inactivation if the nicotine is dialysed away soon after mixing. The extent of inactivation during exposure for a few hours to nicotine at 2 g/l or less is not consistently outside the range of uncertainty of infectivity assays. Effects such as this are probably of no physiological significance because the concentration of nicotine in the leaves that

we use is smaller than this. Commercial experience suggests that tobacco sap can contain as much as 4 g of nicotine per litre. Nicotine was measured on several batches of the leaves we use, by distilling extracts in the presence of MgO, with intermittent addition of water, until the distillate was neutral, and measuring light absorption at 260 nm. No batch of undiluted sap containing more than 0.5 g/l was found. Most of the extracts used in the preceding paper were diluted two- to four-fold and dialysed; this suggests that the nicotine in them would have a negligible effect on the infectivity of TMV:RNA.

TABLE 2. THE EFFECT OF NICOTINE IN VARIOUS ENVIRONMENTS ON THE INFECTIVITY OF TMV:RNA

In four of these tests the TMV:RNA was 60 mg/l for 1 h, but all were 15 mg/l after dilution for assay. Similarly, six of them contained 2 g/l nicotine at the time of assay, and five contained 10 g/l phenol. The nicotine solution was neutralized with acetic acid before use. All operations were at 0 °C, and all the solutions finally contained 5 mM K_2HPO_4.

treatment	lesions on eight half leaves
TMV:RNA in 5 mM K_2HPO_4 for 24 h	204
1 h exposure to 8 g/l nicotine followed by 23 h after dilution, × 4	59
24 h exposure to 2 g/l nicotine	205
1 h exposure to 8 g/l nicotine in a solution that also contained 40 g/l phenol followed by 23 h after dilution, × 4	1
As above, but the mixture included 20 mM K_2HPO_4	0
As above, but dialysed for 23 h against 5 mM K_2HPO_4 before dilution	0
24 h exposure to 2 g/l nicotine and 10 g/l phenol	52
As above, but the mixture included 5 mM K_2HPO_4	168

The position is somewhat different when phenol is present as well as nicotine. A gummy precipitate separates when nicotine is added to water saturated with phenol. Two-thirds saturated phenol precipitates at room temperature when the nicotine concentration is 5 to 6 g/l; 8 g/l is needed at half saturation; the critical concentrations of both components are smaller at 0 °C and in the presence of salts such as K_2HPO_4. In conditions approaching those in which there is precipitation, nicotine and phenol modify purified TMV:RNA in a manner that is not reversed by dialysis. Table 2 shows some aspects of these phenomena. It is clear that dilute nicotine and phenol have little or no effect, and that fourfold dilution after TMV:RNA has been exposed to more concentrated solutions does not reverse the inactivation, nor is it reversed by dialysis. The infectivity is also removed from

phenol-containing leaf extracts when their nicotine content is increased. To get complete inactivation it is necessary to add 4 g of neutralized nicotine per litre, at 2 g/l inactivation is substantial but incomplete, and at 1 g/l it is equivocal. Unless the nicotine and TMV occur close together in the leaf, so that TMV:RNA is exposed to a greater than average concentration of nicotine at the moment when it is split off from TMV protein, it seems unlikely that the co-action of nicotine and phenol is responsible for the differences we find in the infectivity of extracts.

We have no evidence about the nature of the change taking place when TMV:RNA is inactivated by mixtures of nicotine and phenol except that adsorbability seems to be increased. In mixtures that cause inactivation and in which two phases are present, e.g. leaf fibre or a layer of phenol, TMV:RNA is removed from the aqueous layer. This is demonstrated, with mixtures that do not contain leaf extracts, by measuring light absorption at 260 nm on the aqueous phase after repeated distillation *in vacuo* to remove phenol and nicotine, and by using TMV:RNA labelled with ^{32}P with mixtures containing other material that absorbs u.v. light.

Spermine

Because of the possibility that an unidentified base, which Markham found in TMV and later (Johnson & Markham 1962) identified as $NH_2(CH_2)_3NH(CH_2)_3NH_2$, might protect TMV:RNA from inactivation, we (Bawden & Pirie 1959) tried the effects of spermine, spermidine and putrescine on TMV:RNA. At 200 mg/l spermine greatly diminished the number of lesions, and diminished the number slightly at 20 mg/l. This then seemed of little physiological importance because spermine was not known to occur in tobacco. However, spermine and its oxidation products have now been implicated in many reactions involving nucleic acids, and Smith (1970) found 8 mg/kg in tobacco leaves. It is possible therefore that it affects the infectivity of leaf extracts.

Spermine precipitates TMV:RNA from neutral salt-free solution. Precipitation is not visible within a few hours in solutions containing 1 g/l TMV:RNA and 20 mg/l spermine either in the presence or absence of 10 mM K_2HPO_4, but there is aggregation because the TMV:RNA is almost completely sedimented in 90 min at 80000 *g*, whereas sedimentation is only partial in the absence of spermine. The sedimented material is infective when redissolved in 10 mM phosphate and the infectivity of solutions rendered non-infective by spermine is restored by dialysis. There is less loss of infectivity in the presence of electrolytes at concentrations in the range characteristic of leaf extracts. These effects suggest that in dilute solutions of TMV:RNA and spermine there is reversible aggregation so that the number of infective particles in the inoculum is diminished.

As with Ca^{2+} and nicotine, conditions are different when a phenol phase is also present. After dialysis, the aqueous layer from a mixture containing 0.5 g/l TMV, 40 mg/l spermine, 5 mM K_2HPO_4 and 1/5 volume of phenol was not infective, whereas when 50 mM phosphate is present, TMV:RNA moves into the aqueous phase. This movement was confirmed by measurements of light absorption at

260 nm on solutions extracted twice with phenol (to ensure more complete removal of protein from the aqueous layer) and on similar mixtures starting with TMV:RNA rather than TMV.

In the presence of 10 mg/l spermine or less, i.e. at about physiological concentrations, we found no unequivocal evidence for the removal of TMV:RNA from the aqueous phase in the presence of salts, and therefore do not think that spermine is an important agent affecting the infectivity of leaf extracts.

Ribonuclease

Purified leaf RNase is destroyed by phenol. When we started detailed investigation of the infectivity of extracts from infected leaves to which phenol was added at different stages, reported briefly at the International Biochemical Congress in 1964, we did not therefore think that RNase was responsible for the differences in infectivity. This opinion was reinforced by the observation that the diffusate, even after boiling, diminished the number of lesions given by TMV:RNA. Purified leaf RNase is more stable than many enzymes, but is less stable than the pancreatic enzyme (Holden & Pirie 1955). The ability of diffusates to diminish the number of lesions given by TMV:RNA is largely the consequence of the presence in them of Ca, and possibly nicotine, spermine and other small molecules but after some fractionation, diffusates yield an inactivator. When sufficiently concentrated, RNase activity can be demonstrated in it by conventional methods. Only a small part of the total RNase activity of even phenol-treated tobacco leaf extracts is in the initial diffusate and the factors, e.g. turgor in the sac (cf. Craig 1964), and facilitation of passage through it in the presence of phenol and other components of crude leaf extracts, have not been explored. Some properties of the heat and phenol stable enzyme, which is probably a complex with another macromolecule, are described elsewhere (Jervis & Pirie 1972).

Extracts made by pulping tobacco leaves in the presence of one-fifth their weight of phenol and removing the material that can be precipitated by NH_3 and oxalic acid, inactivate TMV:RNA when 2 mg of their dry matter is present in the 0.4 ml used in the standard conditions of inactivation tests. So little inert material is extracted when intact leaves are frozen in ten times their weight of water for 2 or more days and thawed, that 0.02 mg of the dry matter in extracts, cleared in the same way with NH_3 and oxalic acid, causes the same inactivation. Diffusates from these extracts are effective at 2 mg, and, after fractionation by extraction into phenol and precipitation with ammonium sulphate, at 0.01 mg. The most highly purified preparations are active at 1 μg. Leaf RNase that has not been treated with phenol is still more active – but this is not relevant in the present context.

Measurement of the loss of infectivity of TMV:RNA may seem an elaborate and imprecise method for detecting RNase, but it is about 1000 times as sensitive as methods depending on loss of acid precipitability, and it can be used with extracts that are strongly coloured and/or precipitate when acidified. The duration

of contact affects inactivation in the expected manner: thus the number of lesions was diminished from 940 to 705, 445 and 11 after exposure to an enzyme preparation for 20 min, 1 h and 3 h at 0 °C. The effect of different concentrations of enzyme is dramatically nonlinear. Thus in conditions where 1 μg of a preparation had no unequivocal effect, lesions were diminished to 1/10 by 2 μg and to 0 by 3 μg. Conditions in this method for studying an enzyme action are unusual; enzyme and substrate are extremely dilute, and (presumably) breakage of a single bond in a macromolecule, or possibly the mere formation of the enzyme:substrate complex, is the criterion of action.

The phenol-stable RNase complex in leaf extracts is an artefact without physiological significance and its study supplies no information about the total RNase activity in living leaf. But its properties are relevant in the study of leaf extracts in which TMV:RNA, in spite of its vulnerability, retains infectivity. Six factors seem to be important. (1) TMV is disrupted, and most of the protein in a leaf extract is precipitated, by less than full saturation of the aqueous phase with phenol. But the phenol-labile RNase is not then fully inactivated. (2) The stabilized enzyme is partly extracted into the phenol phase (Jervis & Pirie 1972) so that much of it, though not destroyed, is removed from the RNA-containing water phase if enough phenol is used during initial extraction and re-extraction. (3) At pH 8, absorption of TMV:RNA by leaf fibre is minimized and the enzyme is far from its optimum. (4) Because the apparent rate of destruction of TMV:RNA is not proportional to enzyme concentration, extensive dilution during extraction is advantageous. (5) Phenol-stable RNase has little or no activity in solutions saturated with phenol; extracts from infected leaves that are being retained for confirmatory assays should therefore be stored frozen and undialysed. (6) TMV:RNA is protected from attack by RNase by other forms of RNA if present in vast excess. Thus, in a test in which 10 μg of a partly purified enzyme preparation diminished the number of lesions given by 16 μg of TMV:RNA from 112 to 5, the number of lesions when 1.0, 0.3 and 0.1 mg of yeast nucleic acid was present were 152, 100 and 22. No difference was observed in the behaviour of commercial crude and 'highly polymerized' yeast nucleic acid. Normal leaf RNA is probably yet another factor tending to preserve the TMV:RNA in leaf extracts.

Discussion

When an extract is made from infected leaves, virus is released into an environment different from that within infected cells or in the fluids through which infection spreads in the host. As we comment in the accompanying paper: all extracts are artificial, and extracts made in the presence of phenol may be less artificial than others because phenol inhibits some autolytic and oxidative changes. The main reason for differences in the infectivity of extracts made in different ways from the same batch of leaves, is fixation of TMV-RNA on to the leaf fibre. There is also the possibility of inhibition, precipitation and inactivation by other

leaf components. Some possibly relevant substances were not studied in any detail because, though in some circumstances they may be important in the physiology of virus infection, it did not seem reasonable to invoke them in the particular phenomena we were exploring. There is, for example, more chlorogenic acid in young than old tobacco leaves, but differences in the infectivity of extracts are greatest in mature leaves. And 2-hexenal, though it inactivates TMV:RNA as other aldehydes do, becomes more abundant after pulping leaves (Major, Marchini & Boulton 1963); that is, in circumstances leading to the more infective leaf extracts.

The most obvious reason for differences in the behaviour of TMV:RNA, once it is separated from the fibre, in different types of extract, is destruction by leaf RNase. By chemical methods of enzyme assay this action may be overlooked because exposure leading to no detectable loss of precipitability leads to complete loss of infectivity (Bawden & Pirie 1957). During the course of extraction with phenol, part of the leaf RNase becomes stable in the presence of phenol – but it is not enzymically active in water saturated with phenol. Had that not been so it is unlikely that the technique of pulping leaves with phenol would ever yield infective extracts.

In certain environments, nicotine, spermine and Ca^{2+} precipitate both TMV and TMV:RNA. In fluids similar to those produced when tobacco leaves are pulped, the concentrations needed for these precipitations are unphysiologically great. It is, however, probable that these substances are not distributed uniformly throughout the cell; if it should happen that regions of concentration are regions where TMV or TMV:RNA are also concentrated, reactions with these substances could affect the infectivity of extracts. The presence of inclusion bodies and intracellular crystals is a well-known feature of some virus infections. These structures obviously (Pirie 1950) contain material other than virus or they would not be insoluble. We suggested that they might be analogous to the precipitates formed in mixtures of TMV and clupein (Bawden & Pirie 1937) or nicotine (Bawden & Pirie 1940). Though there is no evidence that a large part of the TMV in an infected cell is coupled to substances such as spermine and nicotine, the possibility of such coupling, which would lead to the juxtaposition of coupling agent and virus, makes it difficult to assess the validity of the argument that these substances are too dilute to have an influence.

In the experiments reported here, the substances being tested were used either in simple solution or added singly to leaf extracts. If any serious attempt were being made to correlate their action with differences in the suceptibility to infection of hosts in different physiological states, their simultaneous effects at concentrations that are plausible *in vivo* should be studied.

References

Bawden, F. C. & Pirie, N. W. 1937 The isolation and some properties of liquid crystalline substances from solanaceous plants infected with three strains of tobacco mosaic virus. *Proc. R. Soc. Lond.* B **123**, 274.

Bawden, F. C. & Pirie, N. W. 1940 The effects of alkali and some simple organic substances on three plant viruses. *Biochem. J.* **34**, 1278.

Bawden, F. C. & Pirie, N. W. 1957 The activity of fragmented and reassembled tobacco mosaic virus. *J. Gen. Microbiol.* **17**, 80.

Bawden, F. C. & Pirie, N. W. 1959 The infectivity and inactivation of nucleic acid preparations from tobacco mosaic virus. *J. Gen. Microbiol.* **21**, 438.

Bawden, F. C. & Pirie, N. W. 1972 Factors affecting the amount of tobacco mosaic virus nucleic acid in phenol-treated extracts from tobacco leaves. *Proc. R. Soc. Lond.* B, **182**, 297.

Burström, H. G. 1968 Calcium and plant growth. *Biol. Rev.* **43**, 287.

Craig, L. C. 1964 Differential dialysis. *Science, N.Y.* **144**, 1093.

Holden, M. & Pirie, N. W. 1955 A comparison of leaf and pancreatic ribonuclease. *Biochem. J.* **60**, 53.

Jervis, L. & Pirie, N. W. 1972 The ribonucleases in tobacco leaf extracts treated with phenol (in the Press).

Johnson, M. W. & Markham, R. 1962 Nature of the polyamine in plant viruses. *J. of Virology* **17**, 276.

Legato, M. J. & Langer, G. A. 1969 The subcellular localization of calcium ion in mammalian myocardium. *J. Cell Biol.* **41**, 401.

Major, R. T., Marchini, P. & Boulton, A. J. 1963 Production of α-hexenal by leaves of certain plants. *J. biol. Chem.* **238**, 1813.

Pirie, N. W. 1950 A biochemical approach to viruses. *Nature, Lond.* **166**, 495.

Pirie, N. W. 1956 Some components of tobacco mosaic virus preparations made in different ways. *Biochemical J.* **63**, 316.

Smith, T. A. 1970 Putrescine, spermidine and spermine in higher plants. *Phytochemistry*, **9**, 1479.

Varma, J. P. & Verma, G. S. 1963 The role of calcium in multiplication of tobacco mosaic virus. *Acta Virologica* **7**, 182.