SOME EFFECTS OF TANNIC ACID AND OF LEAF EXTRACTS WHICH CONTAIN TANNINS ON THE INFECTIVITY OF TOBACCO MOSAIC AND TOBACCO NECROSIS VIRUSES

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The inhibition of infection by tobacco necrosis and tobacco mosaic viruses by tannic acid, and by extracts of raspberry and strawberry leaves, was associated with the precipitation of the viruses. Precipitation and inhibition were reversible, and infective virus was obtained from the precipitate formed between the viruses and tannins. Infectivity was fully restored by diluting mixtures of virus and tannin adequately and partially restored by adding alumina or nicotine sulphate.

Viruses and tannins are thought to form non-infective complexes, in which the virus and tannin components are held together by co-ordinate linkages or hydrogen bonds.

Macerating tobacco leaves infected with tobacco mosaic virus together with raspberry leaves greatly decreased the infectivity of the extracts; adding nicotine sulphate to the mixture of leaves before it was ground increased the infectivity, even though nicotine sulphate alone decreases the infectivity of tobacco mosaic virus. Even in the presence of nicotine sulphate, much of the virus was precipitated by substances from the raspberry leaves.

Extracts of roots of *Fragaria vesca* plants, infected with a tobacco necrosis virus, were more infective when made by macerating the roots with four times their weight of buffer at pH 8 than when made without buffer. Various methods are suggested for facilitating the transmission of viruses from plants that contain tannin.

INTRODUCTION

The effects of naturally occurring tannins on plant viruses have been little studied and the literature largely relates to tannic acid, which Allard (1918) and Stanley (1935) found inhibits the infectivity of tobacco mosaic virus. Thornberry (1935) showed that inhibition depends upon the pH and the concentration of tannic acid. When sprayed on the primary leaves of *Phaseolus vulgaris* L., tannic acid decreased the number of lesions produced by subsequent inoculation with virus, although it had little effect, even at high concentration, when applied immediately after inoculation. Infectivity was partially restored to mixtures of tobacco mosaic virus and tannic acid when gelatin was added, or when the mixture was filtered through a collodion membrane and the non-diffusible fraction diluted in phosphate buffer. Johnson (1941) found that infectivity was restored when the tannin was washed out of agar blocks in which tobacco mosaic virus had been treated with 5 % tannic acid.

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Bawden & Kleczkowski (1945) showed that tannic acid exerts its full effect only when incorporated in the inoculum, and concluded that the inhibitor probably affects the virus and not the host. This conclusion was supported by Hirth (1951), who associated the inhibition of infectivity with the formation of an insoluble complex between tobacco mosaic virus and tannic acid at low pH. The precipitate dissolved and its infectivity increased when alkali was added.

Extracts of various plants which contain tannins inhibit the infectivity of plant viruses (van Schreven, 1941; Bawden & Kleczkowski, 1945; Hirth, 1951). As they appear to decrease infectivity regardless of the identity of the virus and the plant to which they are inoculated, van der Want (1951) has referred to tannins as 'absolute' inhibitors, to distinguish them from 'relative' inhibitors, of the type discussed by Bawden & Freeman (1952) and Gendron & Kassanis (1954), which have some degree of specificity.

Tannins are also probably responsible for some of the difficulties in transmitting viruses from some plants. Bawden & Kleczkowski (1945) showed that extracts from the leaves of raspberry and strawberry contain enough tannins to precipitate not only all the proteins within the plant tissues, but also added protein. As they point out, failure to transmit viruses from strawberry and raspberry mechanically is as likely to result from the tannin content of the hosts as from any intrinsic properties of the viruses.

Many species of plants contain large amounts of tannins (McNair, 1930) and few of the many viruses which cause diseases in these plants have been transmitted mechanically. Moreover, even where mechanical transmissions were possible, difficulty was experienced in obtaining consistent results, although once established on hosts free from tannin the viruses were readily sap-transmissible (Moore, Boyle & Keitt, 1948; Brierley & Smith, 1950; Klesser, 1951; Fulton, J. P., 1952; Fulton, R. W., 1952; Yarwood & Thomas, 1954; Cadman, 1956).

In the experiments described below the precipitation of tobacco mosaic and tobacco necrosis viruses by tannins was closely associated with the inhibition of infectivity, and both effects were reversed by treatments which decreased the effective concentration of tannin in the inoculum.

MATERIALS AND METHODS

The virus-host combinations used all gave countable local lesions, so that effects on virus infectivity could be measured quantitatively. Most experiments were made with the Rothamsted type strain of tobacco mosaic virus in *Nicotiana glutinosa* L. or with a tobacco necrosis virus in the primary leaves of *Phaseolus vulgaris* var. Prince. Purified preparations of virus were used in most experiments, but clarified sap from leaves of infected *Nicotiana tabacum* L. var. White Burley was used in some. The tobacco necrosis virus was isolated from strawberry roots and is serologically related to one obtained by Kassanis (1949) from tulips.

Plants were inoculated by rubbing the upper leaf surface as uniformly as possible with the forefinger wet with inoculum. On warm, sunny days the plants were kept in a shaded humid chamber for 24 hr. after inoculation. To prevent the severe wilting and scorching of the leaves caused by nicotine sulphate the leaves of the test plants were rinsed with water after the inoculations were completed.

At least eight half-leaves were inoculated for each comparison and the different treatments were randomized so that each was inoculated an equal number of times on left and right half-leaves, at each leaf position and on each plant.

The tannic acid used was the B.D.H. product from aphid galls on the leaves of *Rhus semialata* Murr. The preparation is relatively uniform in constitution, although it contains several substances in addition to gallotannin, the principal component (White, personal communication). Samples of other commercial vegetable tannins were supplied by Dr T. White, Director of the Central Laboratories of the Forestal Land, Timber and Railway Co. Ltd., Harpenden.

Extracts of raspberry (var. Lloyd George) and strawberry (var. Royal Sovereign) were prepared by passing leaves through a domestic mincer, and expressing the juice through muslin. Strawberry leaves contain little water and half their weight of distilled water was added before squeezing. The extracts were clarified before use by centrifuging for 10 min. at 10,000 r.p.m.

In experiments on the transmission of tobacco mosaic virus from infected tobacco leaves macerated with raspberry leaves, samples of finely chopped leaves were mixed together, and water or nicotine sulphate was distributed as uniformly as possible over the mixture. This was then put through a domestic mincer and the sap expressed through muslin. Except when stated, the extracts were centrifuged, the supernatant fluids dialysed overnight against distilled water and then inoculated to *Nicotiana glutinosa*.

The *Fragaria vesca* L. plants whose roots were infected with a tobacco necrosis virus were supplied by Dr A. F. Posnette from the East Malling Research Station glasshouses. In experiments with these, the roots of one or more plants were thoroughly washed, divided into samples of equal weight and macerated with the appropriate quantity of phosphate buffer at pH 8. As a test of this sampling procedure the roots of two plants were divided into four equal quantities and each was macerated with the same volume of buffer. The four extracts produced a similar number of lesions in *Phaseolus vulgaris* plants.

Nierenstein (1934) has defined the naturally occurring tannins as substances which combine with and precipitate proteins and alkaloids, but other workers restrict the term tannin to substances which have been shown to tan leather. Saps from the leaves and roots of strawberry and from raspberry leaves each contain one or more substances which precipitate alkaloids and proteins. They are referred to as tannins in this work, although they have not been investigated chemically and may not be tannins in the restricted sense of the term. The ability to give a pre-

cipitate with an equal volume of 1 % cinchonine sulphate or 1 % gelatin was used as a test for tannins. The amount and rate of appearance of the precipitate gave an indication of the relative tannin content of different preparations.

Experimental

Precipitation of virus by tannins

Table 1 summarizes the results of two experiments which show that the infectivity of purified tobacco mosaic virus is not permanently destroyed by treatment with a concentration of tannic acid that completely inhibits infectivity. In Exp. 1, mixing purified virus at 20 mg./l. with an equal volume of 1 % tannic acid gave a precipitate which contained almost all the virus; this dissolved in a volume of water equal to that of the original mixture and was then almost as infective as the untreated control.

TABLE 1. *Recovery of infective tobacco mosaic virus from the sediment precipitated by tannic acid*

| | Lesions per half-leaf | | pH of inocula |
Treatment	Exp. 1	Exp. 2	in Exp. 2
A. 1 vol. virus* + 1 vol. distilled water	90	102	5·3
B. 1 vol. virus* + 1 vol. tannic acid,†	1	0	3·2
10 ml. aliquot of preparation B centrifuged			
C. Sediment in 10 ml. water	85	10	4·3
D. Supernatant made up to 10 ml.,	0·5	0	3·2
5 ml. aliquot of preparation C centrifuged			
E. Sediment in 5 ml. water	—	32	5·3
F. Supernatant made up to 5 ml.	—	6	4·3

* In Exp. 1 purified virus at 20 mg./l. and in Exp. 2 at 100 mg./l.
† In Exp. 1 tannic acid at a concentration of 1 % and in Exp. 2 at 4 %.

A heavier precipitate was produced in Exp. 2, when greater concentrations of virus and tannic acid were used. This precipitate dissolved less readily, but additional virus was released by washing the sediment. In each experiment most of the tannic acid remained in the supernatant fluid after centrifuging, although some was carried down on the sediment and released when this was taken up in water. Tests with cinchonine sulphate showed that, in Exp. 2, each of the preparations C and F contained more tannic acid than preparation E; the occurrence of tannic acid was associated with acid conditions, which favour the inhibition of infection (Thornberry, 1935) and the precipitation (Hirth, 1951) of the virus by tannins. In Exp. 2, the first sediment probably contained too much tannic acid for the virus to redissolve and to restore infectivity fully.

Quebracho and mangrove tannins and the gallotannin in tannic acid are not ionized, so that they will not precipitate purified virus by neutralizing the charges

it carries in neutral solution. Also, they will precipitate tobacco necrosis virus, which is soluble over the pH range in which it is stable.

Infective virus was recovered from the precipitate produced when an extract of strawberry leaves was mixed with diluted sap from a tobacco plant infected with tobacco necrosis virus. Strawberry sap in the inoculum almost completely inhibited infectivity, which was partially restored when the sediment was taken up in a small volume of water.

In the experiments with tobacco mosaic and tobacco necrosis viruses, infectivity was restored after tannin and virus stood together for several hours; longer periods of contact were not tested, and tannins may precipitate and inactivate some viruses irreversibly after a prolonged period. This seems unlikely with tobacco mosaic virus, with which Thornberry (1935) showed that infectivity could be restored after infective sap had been standing with 10% tannic acid for periods up to 15 days.

Effect of diluting mixtures of virus and tannin

When mixtures of tobacco mosaic, tobacco necrosis or cucumber mosaic viruses with tannic acid, strawberry sap or raspberry sap, were diluted, infectivity increased, despite the fact that the ratio of virus to inhibitor remained the same. Table 2 gives the results of two dilution experiments; the first shows that infectivity was fully restored when a non-infective mixture of tobacco mosaic virus and tannic acid was diluted. These experiments were made with sap from infected tobacco plants, but similar results were obtained with purified virus preparations. When mixtures of purified virus and tannins were diluted, infectivity increased as the virus precipitate dissolved.

TABLE 2. *Effect of diluting non-infective mixtures of tobacco mosaic virus and tannic acid*

	Mixture	Lesions per half-leaf produced by inoculating mixture diluted
		1:1	1:4	1:16	1:64	1:128	1:256
Exp. 1	1 vol. infective sap + 4 vol. water	120	—	—	16	—	7
	1 vol. infective sap + 4 vol. 1% tannic acid	1	—	—	9	—	7
Exp. 2	1 vol. infective sap + 4 vol. 1% tannic acid	1	12	53	54	33	27

Macerating tannin-containing infected tissues in buffer or water decreased the concentration of tannins in the expressed sap and correspondingly increased infectivity. For example, grinding the roots of *Fragaria vesca*, infected with a tobacco necrosis virus, together with four times their weight of phosphate buffer at pH 8, gave extracts which contained less tannin and were more infective than extracts made without buffer. To quote one experiment, 3 g. samples of infected

strawberry roots were macerated with 3, 12 or 48 ml. of buffer at pH 8 and the average number of lesions per leaf produced by the various extracts was 1, 13 and 6 respectively. Undiluted root extract damaged the inoculated leaves of the test plants and produced no infections.

Interactions of nicotine sulphate and tannin on infectivity

Dahlia mosaic virus is difficult to transmit by inoculating sap from dahlia to dahlia (Brierley & Smith, 1950). Limasset (1951), however, obtained consistent transmissions with partially purified preparations, made from infected leaves macerated with nicotine sulphate, which was claimed to protect the virus from the effects of host tannins. Thung & van der Want (1951) also found that nicotine sulphate protected tobacco mosaic virus from the usual inhibitory effects of raspberry sap released from leaves macerated with infected tobacco leaves.

In my similar experiments, extracts prepared with nicotine sulphate were always more infective than controls prepared with water, although the size of the effect varied considerably in individual experiments. By combining with and precipitating substances in raspberry sap, nicotine sulphate decreased the amount available to precipitate and inhibit the virus. The virus was not protected completely, however; most of it was precipitated by raspberry sap, even when the extract was prepared in the presence of nicotine sulphate. In one experiment, mixtures of 30 g. of healthy raspberry leaves and 5 g. of infected tobacco leaves were treated with 6 ml. of 10% nicotine sulphate or 6 ml. of water. The centrifuged extract from leaves macerated with nicotine sulphate gave an average of three lesions per half-leaf, whereas the extract with water gave none; the sediments gave an average of thirty-three and thirteen lesions, respectively, when taken up in 6 ml. of water.

It is impossible to set adequate controls in experiments on the transmission of virus from mixtures of infected tobacco leaves with raspberry leaves, so subsequent experiments were made with mixtures of leaf saps. In this way the independent effects of nicotine sulphate and raspberry sap were determined. The results summarized in Table 3 show that nicotine sulphate in the inoculum inhibits

TABLE 3. *The interaction between tobacco mosaic virus,*
nicotine sulphate and raspberry sap

Mixture	Subsequent treatment		Lesions per half-leaf
1 vol. infective sap 1:25 + 1 vol. water	2 vol. water added		62
1 vol. infective sap 1:25 + 1 vol. 40% nicotine sulphate	2 vol. water added		33
1 vol. infective sap 1:25 + 1 vol. water	2 vol. raspberry sap added and mixture centrifuged	Supernatant fluid	3
		Sediment	41
1 vol. infective sap 1:25 + 1 vol. 40% nicotine sulphate	2 vol. raspberry sap added and mixture centrifuged	Supernatant fluid	13
		Sediment	12

infection by tobacco mosaic virus and that it only partially protects this virus from precipitation when raspberry sap is added.

Nicotine sulphate also combines with and precipitates tannic acid, and Table 4 shows how nicotine sulphate and tannic acid interact to affect the infectivity of tobacco mosaic virus. Tannic acid in the inoculum almost completely inhibited infections, but inhibited less when the preparations contained nicotine sulphate. Inhibition was least when tannic acid was mixed with nicotine sulphate before adding to the virus, and some infectivity was regained when nicotine sulphate was added to a non-infective mixture of virus and tannic acid. The latter preparation was more infective than a preparation made by adding tannic acid to a mixture of virus and nicotine sulphate, perhaps because tannic acid affects infectivity reversibly, whereas nicotine sulphate has been stated to inactivate virus permanently (Fukushi, 1930).

TABLE 4. *The interaction between tobacco mosaic virus,*
nicotine sulphate and tannic acid*

Mixture	Subsequently added	Lesions per half-leaf		
		Exp. 1	Exp. 2	Exp. 3
1 vol. infective sap 1:20 + 4 vol. water	—	121	85	73
1 vol. infective sap 1:20 + 4 vol. 5 % nicotine sulphate	—	87	69	—
1 vol. infective sap 1:20 + 4 vol. 0·5 % tannic acid	—	3	2	—
1 vol. infective sap 1:20 + 2 vol. 1 % tannic acid	2 vol. 10 % nicotine sulphate	23	9	12
1 vol. infective sap 1:20 + 2 vol. 10 % nicotine sulphate	2 vol. 1 % tannic acid	5	3	9
2 vol. 1 % tannic acid + 2 vol. 10 % nicotine sulphate	1 vol. infective sap 1:20	46	26	18

* Preliminary experiments showed that the inhibitory effect of 1 % tannic acid was minimal after treating with an equal quantity of 10 % nicotine sulphate before adding to the virus.

In similar experiments with the tobacco necrosis virus, infectivity was also restored by adding nicotine sulphate to a non-infective mixture of virus and tannic acid. Tobacco necrosis virus was more affected by nicotine sulphate than was tobacco mosaic virus, and preparations containing nicotine sulphate and tannic acid had greater infectivity than those containing nicotine sulphate alone.

Interaction of alumina and tannin on infectivity

Tannins are strongly adsorbed by alumina and a 1 % solution of tannic acid which had been treated for an hour with alumina at the rate of 200 g. per litre, did not precipitate gelatin and had little effect on the infectivity of tobacco mosaic virus.
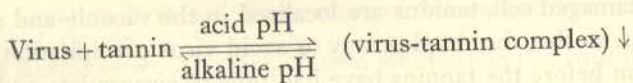
Alumina also had an effect when added to non-infective mixtures containing 0·5 % tannic acid and purified tobacco mosaic virus at 10 mg./l. When 2 ml.

aliquots of this mixture were shaken with 0·1, 0·2, 0·4 or 0·8 g. of alumina, detectable tannin decreased with increasing amount of alumina added, and the preparations had 12, 29, 46 and 56% respectively of the infectivity of comparable inocula which were not treated with alumina or tannic acid.

Certain forms of alumina facilitate virus infection when incorporated in the inoculum (Costa, 1944; Kalmus & Kassanis, 1945), but the granular preparation used in this work (B.D.H., 'aluminium oxide for chromatographic adsorption, for preparatory work') decreased the number of lesions produced by the tobacco necrosis virus on French bean. In the same experiment, a similar quantity of alumina increased the infectivity of comparable inocula which contained 0·5% tannic acid. That alumina did this by acting on the tannic acid and not as an abrasive on the leaf was also suggested by experiments with other abrasives which have little affinity for tannins. Carborundum and 'Celite' greatly increased the number of lesions produced by a control inoculum of tobacco necrosis virus, but had no effect on infectivity when added to non-infectious mixtures of this virus and tannic acid.

DISCUSSION

Hirth (1951) considered that tannins combine with tobacco mosaic virus and it seems likely that this combination resembles the one between hide proteins and tannins. White (1956) summarized the modern literature on this tanning process, and there is general agreement that tannins are fixed by hide proteins because co-ordinate linkages or hydrogen bonds form between reactive groups on the tannin and hide-protein molecules. A relatively weak and dissociable combination of this type between virus and tannins is consistent with experimental observations on the precipitation of tobacco mosaic and tobacco necrosis viruses by tannic acid. These may be summarized by the reversible equation

$$\text{Virus} + \text{tannin} \underset{\text{alkaline pH}}{\overset{\text{acid pH}}{\rightleftharpoons}} \text{(virus-tannin complex)} \downarrow$$

Formation of the virus-tannin complex is favoured by increase in concentration of tannin and by acidity, whereas the complex dissociates on dilution, or by increasing the pH.

The combination of tannins or other inhibitors with viruses does not necessarily account for their effect on infectivity, and only some of the substances which reversibly affect the infectivity of tobacco mosaic virus, precipitate it *in vitro*. This has led Bawden (1955) to suggest, as a general hypothesis, that inhibitors act not on the virus but on the cells of the host, changing them so that they no longer support virus multiplication. The effect of such varied inhibitors as ribonuclease, a glyco-protein from *Phytolacca esculenta*, trypsin and globin may be explained in this way, and these substances have certain features in common. They do not obviously damage the host plant; infectivity is inhibited immediately they are mixed with

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virus solutions, and it is restored partly or wholly by diluting the mixtures or removing the inhibitor.

Tannins differ from inhibitors of the type discussed by Bawden (1955) in that they do not always exert their full effect immediately they are added to a virus preparation, and under certain conditions 5% tannic acid has little effect on infectivity when present in an inoculum of tobacco mosaic virus (Thornberry, 1935). Furthermore, unlike trichothecin, ribonuclease and some other inhibitors, tannic acid has no effect on infectivity when rubbed on the leaf surface immediately after inoculation, and a preliminary treatment with tannic acid has little effect if the leaf surface is washed before inoculation (Bawden & Kleczkowski, 1945). No specific effects such as have been noted with other inhibitors (Stanley, 1934; Bawden & Freeman, 1952; Bawden & Kassanis, 1954) have been found in this or other work with tannins, which have reduced the infections by all viruses to which they have been added irrespective of the host inoculated.

Although my experiments show that tannins resemble other substances in certain aspects of their reversible effect on infectivity, they do not conflict with the view expressed by Bawden & Kleczkowski (1945) and Hirth (1951) that the tannins act directly on the virus. It is suggested that the precipitation of viruses by tannins results in the formation of a non-infective complex, from which infective virus is released by treatments which result in dissociation.

The results presented in this paper suggest methods for increasing the efficiency of virus extraction, by preventing or reversing the precipitation of proteins which normally occurs on macerating tissues containing tannins. The latter are not uniformly distributed throughout the plant and their concentration may vary with season (Skene, in Nierenstein, 1934). It follows that viruses will be transmitted most readily from plants containing tannins by selecting tissues with a high virus content but the minimum amount of tannin.

In the undamaged cell, tannins are localized in the vacuole and separated from the protoplast, so that the simplest way to avoid virus precipitation is to complete the inoculation before the tannins have had time to accumulate and combine with the proteins released when leaves are macerated. This may explain why Yarwood & Thomas (1954) and Yarwood (1955) were able to transmit an apple mosaic and other fruit tree viruses, to herbaceous hosts using a rapid inoculation technique (Yarwood, 1953). Where it is important to decrease the precipitation of proteins by tannins during sap extraction, this can be achieved by macerating the tissues in buffer or water.

With viruses which are reversibly precipitated by the tannins of their host, infective virus may be recovered from the sediment obtained by centrifuging expressed sap. In this way Bennett (1955) obtained infective preparations of curly top virus from the precipitate which forms rapidly in expressed sap from the young leaves of infected water pimpernel (*Samolus parviflorus* Raf.). Tannins may well have been involved in this effect, although tests for their presence were not

described and the precipitation of virus was considered to be due to adsorption on unstable host materials.

The ability of nicotine sulphate to facilitate the transmission of virus from plants that contain tannins has been noted by Limasset (1951) and Cadman (1956), and the technique will probably have further application. The method may be refined by determining the optimum amount and concentration of nicotine sulphate to be used, and it is possible that other alkaloids will be found which combine with host tannins and have less effect themselves in decreasing virus infectivity. Alumina or other substances which adsorb tannins may also be of value in decreasing the effects of host tannins, and preparations may be found which specifically adsorb tannins and at the same time facilitate virus infection.

Viruses have proved difficult to transmit from herbaceous hosts, which do not contain tannins, to susceptible hosts which do. This suggests that the transmission of viruses to and from hosts which contain tannins is complicated by factors other than the difficulty of preparing infective extracts. I have not studied these factors, but the resistance to infection by sap inoculation of leaves which contain tannins may result from the release of these materials from the epidermal cells at the time of inoculation.

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