solution, having been cooled, is centrifuged for 2 or 3 min. to remove any traces of filter paper, and should then be dialysed for 2 days in the refrigerator against frequently changed distilled water. An inactive precipitate may form which can be eliminated by centrifuging. The enzyme solution is now colourless and has a Q_{ACh} of 400,000–600,000.

The yield of this purified preparation amounts to about 20 % of the original activity of the pancreas, but is much greater (40 %) when calculated on the basis that only the albumin fraction is used for purification.

Since enzyme preparations with a Q_{ACh} above 10,000 are adversely affected by salts and dilution with water,* they should be diluted, and their activity measured, in a solution of 0·25 % gum acacia.

* Effects of salts and dilution with water will be discussed in a subsequent paper dealing with the properties of the purified enzyme.

SUMMARY

A simple method is described by which a tissue esterase, capable of hydrolysing acetylcholine, may be purified from dog pancreas. The final preparation, 1 mg. of which hydrolyses about 70 mg. of acetylcholine per minute, is 2000 times as active per unit dry weight as the original material and about 15,000 times as active as horse serum.

The enzyme is a pseudo-cholinesterase, since it hydrolyses esters other than those of choline, and splits acetylcholine with maximum speed at a substrate concentration (0·02 *M*) far above physiological range.

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Methods for the Purification of Tomato Bushy Stunt and Tobacco Mosaic Viruses

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Since we first described methods for making liquid crystalline preparations of tobacco mosaic virus [Bawden & Pirie, 1937] and crystalline preparations of tomato bushy stunt virus [Bawden & Pirie, 1938], we have made preparations of both viruses by many other methods. As a result, we can now describe simpler and better procedures. Other workers have used differential ultracentrifugation; because of the infrequency with which stable substances of high molecular weight seem to occur in normal leaves, this method of preparation is undoubtedly of great value. The handling of large volumes of sap in the ultracentrifuge, however, is laborious, and many laboratories where plant viruses are studied do not have this equipment. Methods of preparation that are trustworthy and involve only ordinary centrifuges may therefore be of more general use.

Tomato bushy stunt virus

In our earlier method the sap was heated to 60° to coagulate the normal leaf protein, and if the preparation is needed solely for a study of its serological or physical properties this is still probably the simplest procedure. However, heating to this extent greatly reduces the infectivity of the virus [Smith, 1935; Stanley, 1940], apparently without affecting its other properties appreciably, and more infective preparations can be made in other ways. The method described below is the most satisfactory we have found, but with small quantities of sap, precipitation with alcohol, as used by Pirie, Smith, Spooner & McClement [1938] for tobacco necrosis viruses, is also suitable and quicker.

Preparation. Leaves and stems of infected tomato plants are put through a domestic meat mincer with $\frac{3}{16}$ in. holes in the plate, and sap is expressed from the mince by hand through a bag of thin, closely woven cloth such as madapollam. For each 100 ml. of sap expressed, 30 ml. of a 4 % solution of anhyd. Na_2HPO_4 is added to the residue in the bag; when this has soaked in, the residue is again put through the mincer and the sap is expressed as before. The two extracts are mixed, centrifuged and the precipitate discarded. The supernatant fluid is usually clear and brown,

but it sometimes has a greenish tinge; this can be removed by adding some more phosphate solution. $(NH_4)_2SO_4$ is added (280 g./l.), and after it has all dissolved the fluid is left for about 12 hr. at room temperature. The precipitate is then centrifuged off, and suspended evenly in a volume of water equal to about one-tenth of the original sap. The fluid is then centrifuged and the precipitate extracted twice or thrice more with smaller volumes of water. The three supernatant fluids are mixed, acetic acid is added to bring the *p*H to 4, the bulky precipitate is centrifuged off, and extracted twice with water. All subsequent operations are carried out at *p*H values between 4 and 4·5.

$(NH_4)_2SO_4$ is added to the mixed acid fluids until there is a definite turbidity; it is convenient to add the solid until about 15 g./100 ml. have been added and then to continue with a saturated solution. Within a few hours at room temperature a precipitate separates from the turbid solution. This can be centrifuged off after about 6 hr. and contains most of the virus. After standing for a few days, however, or after the addition of a little more salt, a further precipitate containing a little virus may separate from the supernatant fluid. This is most conveniently worked up separately. The precipitate is extracted two or three times with dilute acetate buffer so that the volume of the combined extracts is about one-hundredth of that of the original sap. The extract should be colourless or light brown, and only slightly opalescent. At this stage, if old plants have been used, the fluid may still be dark brown; it should then be reprecipitated with $(NH_4)_2SO_4$ and the precipitate, suspended in the minimum amount of water, should be dialysed. As dialysis proceeds a heavy brown precipitate separates; this is centrifuged off, washed once and the fluid used for the extraction of precipitates obtained at a later stage. Saturated $(NH_4)_2SO_4$ solution is now added drop by drop with thorough mixing at a temperature between 20 and 25° until the fluid becomes permanently turbid. Solutions containing more than 1% of virus will begin to crystallize when the $(NH_4)_2SO_4$ concentration has been raised to 10%, but 15% will be needed if the virus concentration is as low as 1 g./l. A precipitate separates after 1–2 min. but dissolves again almost completely when the mixture is cooled to 0°. After 2–3 hr. at 0° any material that remains undissolved is removed by centrifuging at 0°. This can be done in a refrigerated centrifuge or by putting the centrifuge inside a refrigerator. It can, however, conveniently be done in an ordinary machine at room temperature by use of the device illustrated in Fig. 1. A wooden block and lid are turned to fit easily inside the 250 ml. bronze bucket of an International centrifuge and a hole is bored in the block to contain a 50 ml. glass or metal centrifuge tube. If the upper surface of the lid lies a few mm. below the rim of the bronze bucket there is no risk of its being blown off when the centrifuge is spinning. The virus from 1 l. of sap can conveniently be held in four $3 \times \frac{3}{8}$ in. test-tubes, and these are carried, surrounded by ice and water, in the 50 ml. tube. In the model that we use the wooden walls and lid are 12 mm. thick, and some ice remains unmelted after 45 min. centrifuging at 2000 r.p.m. The precipitate can generally be packed tightly by 15 min. centrifuging; if so much $(NH_4)_2SO_4$ has been added that an appreciable amount of virus has also precipitated, it is well to extract the precipitate with water and repeat the precipitation at 0° on the extract.

At this stage the supernatant fluid should be clear and colourless; when left at 0° crystallization will start in a few hours, but if it is allowed to warm up there will be rapid separation of an amorphous product. More virus can be separated in the first fraction if, after a few days, another drop of saturated $(NH_4)_2SO_4$ solution is added for each ml. of fluid. Most of the crystals form on the walls of the tube and, after 4–7 days, the mother liquor can be poured off without centrifuging. If the addition of $(NH_4)_2SO_4$ has been gauged accurately only a tenth of the virus will remain in the mother liquor; this precipitates when the fluid is allowed to warm up and can be centrifuged off, redissolved and crystallized. The crystals do not dissolve completely unless they are left in water for about an hour,

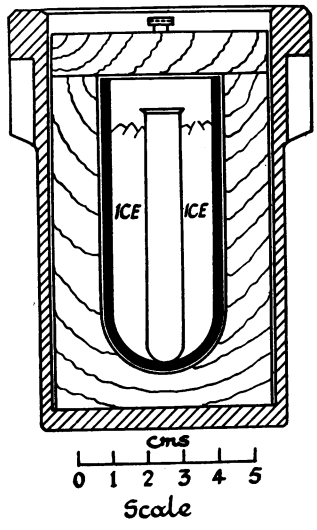

Fig. 1.

and a useful separation from residual contaminants can be effected by washing them quickly. When they have dissolved, any insoluble material is removed, by centrifuging, either immediately or after dialysis. Crystallization seems to proceed equally satisfactorily at any *p*H between 3 and 7, but the removal of traces of denatured virus and normal leaf protein is easiest at about *p*H 4·5.

One variant of this method may on occasion be useful. The addition of enough acid to bring the *p*H of the original sap to 4·5 removes more normal protein than the addition of phosphate and leads to no greater loss of virus. It does not, however, remove so much of the Ca from the sap, and this, if not removed, precipitates with the $(NH_4)_2SO_4$ and may interfere with the subsequent extraction of the virus from the first $(NH_4)_2SO_4$ precipitate. If the leaves that are being used in a virus preparation happen to have a low Ca content, there may be greater advantages in getting rid of the normal protein than in getting rid of the Ca, and this variation may make the preparation go more smoothly.

The C, H and N content of a virus preparation gives but little information about its purity; the P content, on the other hand, is a useful guide. In our earlier paper we gave 1·3–1·5% as the range within which the P contents of our preparations fell. In the more extended series of analyses that has since been carried out, on preparations dried at room temperature *in vacuo* over P_2O_5, we find that preparations containing less than 1·4% can always

be further fractionated. Under the conditions used by us the P method of Kuttner & Lichtenstein [1932] has a probable error of 5 %. With this uncertainty we look upon 1·5 % as the most probable value for the P content. An impure preparation of bushy stunt virus is likely to have a low P content, but, as we have already pointed out [Bawden & Pirie, 1938], its carbohydrate content will probably be high. The precipitate that separates from most preparations on prolonged dialysis at about *p*H 4·5 invariably has a high carbohydrate content, and it is rare to find a carefully purified preparation that contains as much as 7 % of carbohydrate estimated by the orcin method [Pirie, 1936] using glucose as a standard. The most usual value is 6 %. It is not possible to assess the significance of the difference between this figure and the range 11·4 to 7·1 % given for the carbohydrate content of centrifugally isolated virus by Stanley [1940], for he does not mention the method of estimation used, and the different methods of carbohydrate estimation give results that are not necessarily comparable.

The only difference we have found between preparations made by the method described in this paper and by that described earlier is that the former are more infective. In appearance, crystallizability, serological activity, analytical composition and sedimentation constant, they are indistinguishable. Recent measurements on preparations made by both methods give an $S_W^{20^\circ} = 130 \times 10^{-13}$. The discrepancy between this figure and that given by McFarlane & Kekwick [1938] is discussed in the Addendum to Bawden & Pirie [1943].

Table 1. *Comparison of activity of bushy stunt virus prepared by two methods*

Test	Method	Serological titre	Infectivity Av. no. of lesions per leaf at 10^{-4}	Infectivity Av. no. of lesions per leaf at 10^{-5}
1	Heating	1/600,000	35	9
	No heating	1/600,000	105	45
2	Heating	1/600,000	26	4
	No heating	1/600,000	96	40

In Table 1 purified preparations made by the two methods are compared, and it will be seen that virus made by the method described in this paper is at least 10 times as infective as virus made by the method involving heating. It is more difficult to be certain that the method causes no loss of infectivity. Comparisons between the infectivity of sap and the purified virus suggest that there is no loss, but it is well known that the constituents of sap can affect the number of lesions produced. The extent of this depends at least in part on the ratio of virus to normal plant constituents. This is illustrated in Table 2; during one purification the removal of contaminants has given an apparent increase in infectivity whereas in a second, with much more infective sap, it has not. To get the most highly infective preparations, sap should be worked up immediately, for, as we have already shown [Bawden & Pirie, 1940], during ageing *in vitro* the virus loses infectivity in much the same way as when heated.

Table 2. *Comparison of activity of sap and purified bushy stunt virus preparations*

Preparation	Serum precipitation end-point	Av. no. of lesions per leaf at 1:20	Av. no. of lesions per leaf at 1:200
Preparation 1:			
Sap from stalk and leaves	1:12	44	7
Virus preparation diluted to original vol. of sap	1:12	67	20

		Av. no. of lesions per leaf at 1:10	Av. no. of lesions per leaf at 1:100	Av. no. of lesions per leaf at 1:1000
Preparation 2:				
Sap from stalk and leaves	1:160	186	86	8
Virus preparation diluted to original vol. of sap	1:120	188	86	6

The virus content of the leaves from infected tomato plants is at least five times as great as that of the stems; the inclusion of stems in the preparation therefore has only a small effect on the yield and increases greatly the labour of mincing. The mincing is easier if the leaves have been frozen beforehand, but this freezing has no other advantage. We have, however, no evidence that it causes any loss of virus, for the salts and other constituents of sap protect the virus from the inactivation that takes place when virus solutions in water are frozen [Bawden & Pirie, 1938]. This phenomenon will be dealt with more fully in the following paper.

We have generally used tomato plants as a source of the virus and the yields have varied from 3 to 100 mg./l. of sap. The highest yields are obtained from young seedlings infected in the winter and the lowest from older plants infected in the summer. Purification is also easier from plants growing during the winter, as the normal plant constituents are then less troublesome to remove. We have also made preparations from *Datura stramonium* and *Nicotiana glutinosa*. *D. stramonium* is recommended by Stanley [1940] as giving a higher yield than tomato, but in our experience the sap of this plant is more troublesome to handle. The yield from *N. glutinosa*, as would be expected from a plant giving local lesions only, is very low. These yields all refer to the sap that is expressed after mincing in a domestic meat mincer. By more thorough grinding of the

leaf residue a further equal quantity of virus can be isolated. This phenomenon will be described in a later paper.

Tobacco mosaic virus

Precipitation with alcohol was one of the first steps in the method we previously described for the purification of tobacco mosaic virus. This successfully removed most of the normal leaf protein but was laborious.

In the preparations carried out during the past few years we have relied on precipitation at *p*H 3·3 to render the normal protein irreversibly insoluble. We have already stressed the aggregation that appears to accompany purification of the virus by our methods [Bawden & Pirie, 1937]; Bernal & Fankuchen [1941] suggest that this is a necessary corollary of purification and that the tendency towards linear aggregation of the particles is increased by the removal of some of the normal components of sap. Our view that preparations of tobacco mosaic virus are not homogeneous and consist of rods of equal cross-section but variable length, has received valuable support from Frampton's measurements [1942] of the lengths of virus particles measured by means of the electron microscope.

Preparation. Sap from infected plants is prepared by mincing and extraction with phosphate solution as in the method used for preparing bushy stunt virus. To each litre of fluid 250 g. of $(NH_4)_2SO_4$ is added and, after about ½ hr., the precipitate is centrifuged off; it is extracted with water until the extracts no longer have an obvious shimmer when stirred. In general four or five extractions are necessary, and the final volume of the extract is about one-quarter of the original volume of the sap. 1% NaOH is added in a fine stream with vigorous stirring to bring the *p*H to about 6·5, and the precipitation with $(NH_4)_2SO_4$ is repeated; if the supernatant is still dark brown a third precipitation is carried out. The precipitate is suspended in 10 times its bulk of water and centrifuged for 1 hr. at 3500 r.p.m., the dark brown precipitate is re-extracted with a smaller volume of water, and the virus precipitated by bringing the *p*H of the combined extracts to 3·3 by the addition of dilute HCl. After ½ hr. this precipitate is centrifuged off, washed once by mixing thoroughly with water and centrifuging again, transferred with the minimum amount of water to a dialysis sac, and dialysed against running tap water. After 24–36 hr. the *p*H will have risen sufficiently for the virus to dissolve, but at this *p*H, and in the presence of only traces of salt, most of the normal protein remains insoluble. The contents of the dialysis sac are diluted if necessary and centrifuged; centrifugation is likely to be very slow if the fluid contains more than 1% of solid. If the precipitate is bulky it should be re-extracted. This method of dissolving the acid precipitate of virus has been adopted because it avoids the risk of inactivating the virus in the small regions of high *p*H which are almost unavoidable if alkali is added directly. This dialysis also brings about a useful separation of the virus from some normal constituents of the sap.

The further treatment depends largely on the appearance of the fluid. If young tomato or white burley tobacco plants have been used it will be nearly colourless, but with older plants it will be brown. If the fluid is brown the *p*H should be adjusted to 6·5 and the $(NH_4)_2SO_4$ precipitation repeated. Pale or colourless preparations are reprecipitated with acid and dialysed thoroughly against distilled water; the dialysis sac should be shaken gently. If necessary the fluid is centrifuged and is then left undisturbed in the ice chest for a few days for the separation of the lower, liquid-crystalline, layer. With aucuba mosaic this should separate from a solution containing 2% of virus, but with the other strains 3% is generally necessary. As in preparations made by the previous method the lower layer is, from a chemical standpoint, the purer and has a higher serum precipitation end-point than the upper, but it is slightly less infective. This method of preparation gives good results with either tobacco or tomato plants.

The recovery of virus by this procedure is satisfactory and there is no evidence of any great inactivation. Thus from 1600 ml. of tomato sap 610 mg. of aucuba mosaic virus was isolated in the form of 'Bottom layer' and 435 mg. as 'Top layer', that is to say 0·66 g. was isolated for each litre of sap. The serum precipitation end-point of the sap was 1:1500. If the 0·66 g. represented complete recovery of virus this corresponds to an end-point of $2{\cdot}3 \times 10^6$, and any loss during the preparation necessitates the assumption of an even lower end-point. The 'Top' and 'Bottom' layers, on the other hand, had serum precipitation end-points of $1:4 \times 10^6$ and $1:6 \times 10^6$ respectively. For infectivity measurements it was assumed that the sap contained 0·66 g./l. and dilutions were made accordingly. In Table 3 the number

Table 3. *Average number of lesions per leaf for sap and purified aucuba mosaic virus*

	Dilution of virus		
	1 : 10^5	1 : 10^6	1 : 10^7
Sap	76	21	1
'Bottom layer'	49	15	0
'Top layer'	52	12	2

of lesions found is set out. These results are most simply explained by postulating that linear aggregation of the virus particles during purification has increased the serum precipitation titre, whereas the fall in infectivity, which would be expected to accompany such aggregation, has been masked by an apparent increase in infectivity due to the removal of normal sap constituents.

The serum precipitation end-points given above for aucuba mosaic virus are similar to the end-points 6×10^6 to 10^7, found for this strain of virus in 1937. With tobacco mosaic virus, on the other hand, we are unable to duplicate the old high values for the end-point. The strains that we handle now give precipitation end-points of about 10^6 instead of the old values of 6×10^6 and 10^7. This change is probably due to the inadvertent selection of a new strain of tobacco mosaic virus, but we have no definite evidence of this.

SUMMARY

Methods, requiring only low-speed centrifuges, are described for the purification of tomato bushy stunt and tobacco mosaic viruses.

These preparations appear to contain virus that is weight for weight as infective as that in clarified sap. There is evidence, however, that the tobacco mosaic virus particles have undergone some aggregation.

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The Inactivation of Tomato Bushy Stunt Virus by Heating and Freezing

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We have often stressed the fact that there is some uncertainty over the exact name most suitable for use when speaking of purified virus preparations. It can hardly be questioned that the specific nucleoproteins, isolated from infected plants, bear some relationship to the viruses themselves, and we have used the term viruses when referring to these proteins. The exact relationship between these proteins and the viruses as they are produced in the infected cells, however, is by no means clear.

All the viruses with which we have worked can be rendered non-infective without changing their serological reactions, and such inactive virus preparations have physical properties at present indistinguishable from those of active virus. Because of this, physical and serological tests cannot be taken as proving the homogeneity of virus preparations. With viruses such as potato '*X*' and tobacco mosaic complete loss of infectivity without loss of serological activity has been produced by X-rays, ultraviolet light, H.CHO, H_2O_2 and HNO_2, but the activity and homogeneity of purified preparations of these viruses is affected by the tendency of the particles to aggregate linearly [Bawden & Pirie, 1937; 1938*b*]. Tomato bushy stunt and tobacco necrosis viruses do not appear to aggregate in this way, but they are more easily rendered non-infective by ageing *in vitro*, moderate heating and treatment with alkali.

In the method we described for the isolation of a crystalline protein from plants suffering from bushy stunt, the sap was heated to about 60° to facilitate clarification [Bawden & Pirie, 1938*a*]. We now know that this treatment must have led to considerable inactivation, and Stanley [1940] showed that more infective preparations could be produced by differential centrifugation. More infective preparations can also be made by precipitation methods if heating is omitted. The method of isolation we describe [Bawden & Pirie, 1943] gives a crystalline product that weight for weight is as infective as the virus in clarified infective sap. At the moment this is the only test available for estimating full activity, but it is not necessarily a valid one. First, it is possible that inactivation without loss of serological activity occurs in the living plant, so that the virus in the sap is already a mixture of infective and non-infective particles. Secondly, sap may contain some inhibitor of infectivity; if this is so, some inactivation could occur during purification but pass unnoticed, as it would be balanced by the removal of the inhibitor. Until more sensitive methods for detecting activity are developed, so that it can be shown that one virus particle can cause infection, it is not likely to be possible to prove that any virus preparation is homogeneous.

The main conclusions of our earlier paper on tomato bushy stunt virus were confirmed by Stanley [1940]. There are some apparent disagreements, however, and the significance of these is discussed in this paper, which deals mainly with the effects of heating and freezing the virus. The methods of testing were similar to those described previously [Bawden & Pirie, 1938*a*].