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.

REFERENCES

Bawden, F. C. & Pirie, N. W. [1937]. *Proc. roy. Soc.* B, **123**, 274.
—— —— [1938]. *Brit. J. exp. Path.* **19**, 251.
—— —— [1940]. *Biochem. J.* **34**, 1278.
—— —— [1943]. *Biochem. J.* **37**, 70.
Bernal, J. D. & Fankuchen, I. [1941]. *J. gen. Physiol.* **25**, 111.
Frampton, V. L. [1942]. *Science*, **95**, 232.
Kuttner, T. & Lichtenstein, L. [1932]. *J. biol. Chem.* **95**, 661.
McFarlane, A. S. & Kekwick, R. A. [1938]. *Biochem. J.* **32**, 1607.
Pirie, N. W. [1936]. *Brit. J. exp. Path.* **17**, 269.
—— Smith, K. M., Spooner, E. T. C. & McClement, W. D. [1938]. *Parasitology*, **30**, 543.
Smith, K. M. [1935]. *Ann. appl. Biol.* **22**, 731.
Stanley, W. M. [1940]. *J. biol. Chem.* **135**, 437.

The Inactivation of Tomato Bushy Stunt Virus by Heating and Freezing

By F. C. BAWDEN and N. W. PIRIE, *Rothamsted Experimental Station, Harpenden, Herts*

(*Received 4 September 1942*)

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*].

EXPERIMENTAL

The effect of heating purified virus preparations

The actual thermal inactivation point of bushy stunt virus, i.e. the temperature at which 10 min. heating gives complete inactivation, has varied in different tests between 70 and 85°. The variation may depend partly on the concentration of the virus solution heated, but it appears chiefly to be determined by the susceptibility of the host plants used. The same inoculum may produce 10 or more times as many local lesions in one lot of test *Nicotiana glutinosa* plants as in another, and it is obvious that the term complete inactivation has no real meaning in these conditions, for whether or not infection is obtained depends first on the concentration of the inoculum and secondly on the susceptibility of the host. In general, the younger the *N. glutinosa* plants the more lesions per leaf are obtained. In moderately developed plants, there is an increase in the susceptibility of leaves from the base to the top so that the same inoculum may only give one or two lesions on a basal leaf but give 50 or 60 on leaves nearer the top. This is in striking contrast to results with tobacco mosaic virus on the same host where there is a gradient of increasing susceptibility from the top to the base. With tobacco mosaic virus the differences are less pronounced, but if solutions of the two viruses are rubbed on opposite halves of the same leaves, those at the top of the plant will often give more lesions with bushy stunt than with tobacco mosaic virus, whereas those at the base will bear great numbers if rubbed with tobacco mosaic but few or none with bushy stunt virus.

Table 1. *Effect of heat on purified bushy stunt virus made with and without previously heating infective sap*

	Virus from unheated sap		Virus from heated sap	
Temp.	Serological titre	Av. no. of lesions per leaf at 10^{-4}	Serological titre	Av. no. of lesions per leaf at 10^{-3}
Unheated	1/600,000	83	1/600,000	78
45°	1/600,000	58	1/600,000	55
50°	1/600,000	33	1/600,000	36
55°	1/600,000	16	1/600,000	19
60°	1/600,000	5	1/600,000	12
70°	1/600,000	3	1/600,000	8
80°	1/600,000	1	1/600,000	4
85°	*Nothing at 1/5000	0	*Nothing at 1/5000	0

* Tested after centrifugation; the infectivity results are expressed as the average number of lesions per leaf on *N. glutinosa*.

Influence of temperature. Table 1 shows the effect of 10 min. heating at various temperatures on two preparations of virus made in different ways, one in which the infective sap was previously heated and the other not. Both were heated as 0·1 % solutions in 0·1 *M* phosphate buffer at *p*H 6, the preparation from heated sap was tested for infectivity without further dilution and the other was diluted 1:10 so that the two were comparable in infectivity. Although loss of infectivity is only complete after heating at 85°, some infectivity is lost at temperatures as low as 50° and most of it below 60°. By contrast, the precipitation titre is unaffected by heating up to 80°, but a little heating above this is sufficient to destroy the serological reactions. This destruction is accompanied by the separation of a coagulum. Thus there is a wide range in the temperatures over which loss of infectivity can be measured, but loss of serological activity resembles other examples of protein denaturation in proceeding at a measurable rate only within a narrow temperature range. In this respect tomato bushy stunt virus resembles the tobacco necrosis viruses and differs from other viruses investigated, which lose both infectivity and serological activity *pari passu* in a narrow temperature range [Price, 1938; 1940; Bawden, 1941]. Virus preparations that have been largely inactivated by heating during the preparation lose their residual activity according to much the same law that governs the loss of activity by fully active preparations.

Influence of duration of heating. Preparations with no demonstrable infectivity can be produced by heating at temperatures well below the thermal inactivation point, but prolonged heating is necessary. Thus in *p*H 6 phosphate buffer at 50°, a 0·5 % virus solution lost half of its infectivity in 15 min. three-quarters in 1 hr. and all in 24 hr.; at 60° the solution was non-infective after 1 hr. and at 70° after 30 min. Such heated preparations do not change their appearance, they retain their full serological activity and crystallize as easily and in the same form as unheated ones.

Table 2. *The infectivity of fractions from a mixture of infective and non-infective sap*

	Av. no. of lesions per leaf at	
Virus preparation	10^{-4}	10^{-5}
1. Active virus	80	41
2. Inactive virus	0	0
3. Mixture of 1 part of no. 1 with 4 parts of no. 2	46	18
4. 1st crystalline fraction from no. 3	43	15
5. 2nd crystalline fraction from no. 3	38	16

We have attempted to fractionate preparations of partially inactivated virus by crystallization but have had no success. As many as five crystalline fractions have been made from preparations derived

from heated sap, but they all had similar infectivities. Artificial mixtures of infective and non-infective virus have also been made and subjected to fractional crystallization, without any separation. The results of one such test are given in Table 2.

Influence of p*H*. The virus is most stable around *p*H 6, but heating at any *p*H value between 4 and 9 can cause loss of infectivity without a corresponding loss of serological activity. At *p*H 4, however, denaturation occurs with much less heating than at higher values. In *p*H 6 *M*/10 phosphate buffer, 0·1 % solutions become opalescent after 10 min. at 80° and still further heating is necessary to cause precipitation. In *p*H 4 *M*/10 phthalate buffer, on the other hand, 0·1 % solutions are opalescent after 10 min. at 60° and 10 min. at 70° causes precipitation.

Heating for 10 min. at 60° and *p*H 4 has no effect on the serological activity, but material that is flocculated specifically by virus antiserum can be removed from such solutions by centrifugation for a few minutes at 5000 r.p.m. Heating for longer periods in these conditions increases the amount of this material, but it also causes the spontaneous separation of a precipitate. At *p*H 6 more intense heating is needed to produce this effect. Table 3

Table 3. *Effect of heating purified bushy stunt virus solutions at* p*H 6 and 80° for various times on the serological activity*

Time of heating min.	Appearance of heated fluid	Serological titre: Before centrifuging	Serological titre: After centrifuging
0	Clear	1/320,000	1/320,000
7·5	Slightly opalescent	1/320,000	1/160,000
15	Opalescent	1/160,000	1/ 40,000
30	Opalescent and slight flocculation	*1/ 80,000	1/ 5,000
60	Considerable flocculation	*1/ 10,000	No ppt. at 1/2,000

* Tests made on supernatant fluid after floccules had settled.

shows the results of heating 0·1 % solutions of virus in 0·1 *M* phosphate buffer at *p*H 6 for various times at 80°.

The effect of miscellaneous treatments. The nature of the changes in the virus particle when it is rendered non-infective by an amount of heating insufficient to cause denaturation is unknown. As treatment with H_2O_2 has a similar effect, it was thought that oxidation might be responsible. Tests were made therefore to compare the effects of heating virus solutions at *p*H 6 in air and *in vacuo*, but no differences were found. In one such test, the unheated control gave an average number of lesions per leaf at 10^{-3}, 10^{-4} and 10^{-5} of 79, 57 and 22, while those heated in air and *in vacuo* for 10 min. at 60° gave 19, 6 and 1·5 and 21, 8 and 2 respectively. Similarly, attempts to preserve the infectivity of bushy-stunt virus in expressed sap by the addition of reducing agents has failed. Indeed, the addition of 5 % Na_2SO_3 caused a rapid inactivation of a type resembling that caused by heating or ageing. Within 24 hr. at room temperature the sap had lost its infectivity, but from it we isolated a crystalline and serologically active product indistinguishable from a normal virus preparation except for its complete lack of infectivity. This type of inactivation is not reflected by any change in the sedimentation constant or in the phosphorus or carbohydrate contents. This is shown in Table 4, where sedimentation constants and analytical figures for virus preparations

Table 4. *Sedimentation constants and carbohydrate and phosphorus contents of active and non-infective purified virus preparations*

		Phosphorus %	Carbohydrate %
Fully active virus	$S_{20} = 130{\cdot}9 \times 10^{-13}$	1·47	6·0
Virus inactivated by heating at 59–60°	$S_{20} = 130{\cdot}1 \times 10^{-13}$	1·44	6·5
Virus inactivated by ageing	$S_{20} = 130{\cdot}2 \times 10^{-13}$	1·53	6·6

made non-infective by various treatments are given. The analytical methods were those used in our earlier paper. Sedimentation constants were measured by Dr A. G. Ogston and are set out in the Addendum to this paper.

The effect of freezing purified virus preparations

We have already briefly noted the inactivation of purified preparations of bushy stunt virus by freezing [Bawden & Pirie, 1938*a*]. These experiments were made only with dialysed solutions containing 5–20 g./l. and near the isoelectric point [*p*H 4·11, McFarlane & Kekwick, 1938]. Stanley [1940] found that freezing infected leaves did not destroy the virus, and we have also found that freezing plant tissues or expressed infective sap does not inactivate. We have now made experiments on the effects of freezing the virus in different conditions and find that the rate and extent of inactivation are affected by a wide range of variables. The variables we have considered are concentration of virus, *p*H, duration of freezing, temperature to which the preparation is taken, and the presence of other materials.

Unless otherwise stated, the virus preparations used in this work were purified and dialysed. So far as we know they were free from any material

other than virus. Some preparations were made by the old method, which involved heating the sap to 60°, and some by the method described in the preceding paper. Several comparisons have been made in which these two products were frozen under the same conditions and no differences have been found in their behaviour.

. No experiments made at temperatures below −20° are reported in this paper. Thus, freezing means the more or less complete separation of crystalline ice, and our experiments have little in common with those [e.g. Luyet, 1937; Luyet & Thoennes, 1938] in which vitrification is induced by sudden, extreme cooling.

All the experiments on freezing were carried out in 3 × ⅜ in. test tubes. The mixture of virus solution and the other components of the system was carefully pipetted in to form a pool at the bottom of the tube without wetting the walls; in general 0·1–0·4 ml. was frozen. A metal stand, previously cooled, held the tubes in a nearly horizontal position in a part of the refrigerator at the required temperature. Freezing generally occurred after a few minutes, but if it did not the mixture was seeded with about 0·005 ml. of ice carried on a cooled rod. In some experiments, especially those in which only short periods of freezing were used, the tubes were partly immersed in an ice and alcohol freezing mixture at the required temperature. This was in a glass vessel so that the moment of freezing could be easily noted. No differences have been detected between mixtures freezing at the slightly different rates that these different procedures probably involve.

After thawing, the amount of insoluble material was estimated by eye; apparently complete precipitation was called 3, substantial precipitation but a still opalescent supernatant 2, and a trace only of precipitate was called 1. The *p*H of the mixture was brought to 5·5 by the addition of *p*H 6·0 phthalate buffer or, in the experiments run at low *p*H's, phthalate to which an excess of NaOH had been added. Before testing for serological activity and infectivity the mixture was diluted with 0·9% NaCl solution so that its virus content, in terms of the initial amount of virus present, was 0·2 g./l., and centrifuged.

Influence of the concentration of virus. Table 5 shows that, at *p*H 4·1 and 2 hr. freezing, inactivation is complete only when the virus concentration exceeds 10 g./l. It also shows that the inactivated virus becomes insoluble. The results here are in good agreement with those of Nord and his collaborators in a series of papers on 'cryolysis', e.g. Nord [1933], Nord & Lang [1935], Holzapfel & Nord [1938 *a*, *b*] and Kausche & Holzapfel [1940]. They find, using a wide range of colloidal materials, that changes leading to an aggregation of the particles are favoured by an increase in the concentration of the solution that is being frozen, and that dilute solutions tend to disaggregate. An aggregation leading, as with bushy stunt virus, to the separation of a precipitate was not observed in Nord's systems, but it is a very well-known result of freezing other systems; soil [Jung, 1931] and normal leaf protein [Bawden & Pirie, 1938*c*] may be mentioned as examples, and the similar behaviour of agar is the basis of the methods used for its commercial preparation.

Table 5. *Effect of freezing purified preparations of virus at* p*H 4·1; influence of virus concentration*

Conc. of virus (g./l.)	Amount of material insoluble after thawing (arbitrary units, see p. 73)	Serological end-point
20·0	3	No ppt. at 1 : 1,000
10·0	3	1 : 1,000
5·0	3	1 : 4,000
2·5	2	1 : 32,000
1·25	1	1 : 128,000
Unfrozen control	0	1 : 320,000

The virus was dissolved in *M*/20 phthalate buffer solution at *p*H 4·1 and the samples were held frozen at −10° for 2 hr.

Influence of p*H.* Although Table 5 shows that concentration is an important factor in determining the degree of inactivation on freezing, *p*H is even more important. When solutions of the same concentration as those listed in Table 5 are frozen at *p*H 3·5, for example, even the most dilute is completely inactivated. Inactivation also occurs in solutions more dilute than these. At *p*H 5, on the other hand, inactivation is incomplete even after 12 hr. freezing. The results of an experiment in which a series of tubes containing 1 g. virus/l. were frozen at different *p*H values are set out in Table 6. It is clear from this experiment that an acid solution favours inactivation.

Table 6. *Effect of* p*H on purified virus preparations*

*p*H	Amount of material insoluble after thawing (arbitrary units, see p. 73)	Serological end-point
3·5	3	Less than 1 : 5,000
4·0	3	1 : 40,000
4·5	2	1 : 80,000
5·0	1	1 : 200,000
5·5	0	1 : 400,000
6·0	0	1 : 400,000
Unfrozen control	0	1 : 400,000

1 g./l. solutions were held frozen at −10° for 12 hr. in *M*/20 phthalate buffer.

Influence of duration of freezing. The time for which the material is held frozen can also affect the extent of inactivation. In general, the amount of inactivation increases with the duration of freezing. Clear-cut results can be obtained when the differences in duration of freezing are large, but, as perhaps might be expected, we have been unsuccessful in getting consistent results when comparing

times of freezing that do not differ greatly from one another. Table 7 illustrates these phenomena at *p*H 4·5; at other *p*H values the extent of inactivation also depends on the duration of freezing. Under the conditions of these experiments all the liquid has apparently solidified within a few seconds of the

Table 7. *Effect of duration of freezing on purified virus preparations*

Duration of freezing (min.)	Amount of material insoluble after thawing (arbitrary units, see p. 73)	Serological end-point	Av. no. of lesions per leaf at 10^{-4}	Av. no. of lesions per leaf at 10^{-5}
0	0	1:320,000	133	57
10	0	1:320,000	131	31
40	0	1:160,000	124	18
225	1	1:160,000	113	28
450	1	1:160,000	47	15
1465	3	1: 40,000	5	1
1930	2	1: 80,000	5	1
2410	3	1: 20,000	3	1

0·1 ml. lots of 10 g./l. virus solution in *M*/30 phthalate buffer at *p*H 4·5 were frozen for the time stated.

first signs of ice formation, for there is considerable supercooling and the volumes of fluid are small, whereas the surface exposed to cold and the heat capacities of the vessel and stand are large. This apparently almost instantaneous solidification, however, does not necessarily imply immediate complete freezing. Moran [1926], by following the volume changes in a gel containing 44 % of gelatine, showed that freezing was only complete after 26 days at −11°, and Kistler [1936] reached a similar conclusion with emulsions of water and toluene. The latter reviews the literature of the freezing of colloids as does Blanchard [1940]. Our mixtures have a much lower colloid content than those used in the experiments of the workers referred to above, and we have no evidence that incomplete inactivation in short exposures to cold is in fact due to incomplete freezing at −10°. However, this is the simplest explanation of our results and may well be adopted until some evidence against it is forthcoming.

Influence of freezing to different temperatures and in the presence of various other materials. There are three obvious ways in which another solute may affect the inactivation of bushy stunt virus by freezing. First, if the eutectic temperature of the substance added is lower than the temperature to which the mass is frozen some of the solution will remain unfrozen; if enough of this solution is available to dissolve the virus, that is to say if the ratio of solute concentration to virus concentration is high enough, there is no reason why the virus should be affected by the freezing. In the second place, it may delay the establishment of complete freezing throughout the mass of the material; if a substance is acting in this way an increase in the duration of freezing should neutralize the effect. Finally, the solute may combine with the virus or precipitate it from solution; the complex or the precipitate may have a different stability from the normally dissolved virus when the surrounding water is frozen. Phthalate buffer was present in all the freezing experiments described in this section, and also in the three described above, in which it was necessary to control the *p*H. The solubility of phthalate is so low that we have no reason to think that it exerts any protective action at −10° nor have any experiments suggested it. All the data on eutectic temperatures and the concentration of the eutectic are taken from Landolt and Bornstein's Tables.

Table 8 illustrates the first mechanism. Probably because of the length of time for which the samples were held frozen these results were clear cut, and the virus was either inactivated or else retained its infectivity and serological activity substantially unimpaired. Table 9 illustrates the first and second mechanisms, KNO_3 delays the inactivation somewhat, and NaCl, in spite of its low eutectic, does not, at this concentration, protect indefinitely. The amount of water that would be held unfrozen at −10° by the 0·27 mg. of NaCl that is in these 0·1 ml. test samples is 0·81 mg. This amount is apparently insufficient for the permanent stability of 1 mg. of virus under these conditions. In another experiment with 10 g. virus/l. at *p*H 4·5, inactivation was not appreciable in 24 hr. at −10° in the presence of 0·56 % NaCl but was nearly complete with 0·17 %. The results in a series of tubes containing the latter mixture were very variable at shorter times of freezing; this suggests that we are here at about the limit at which the amount of water held unfrozen is insufficient to preserve the virus. This hypothesis offers an explanation of the greater stability of dilute virus solutions for, other things being equal and in the presence of unavoidable traces of salt, more water is available in them for each mg. of virus. The protective action of glucose is also shown in Table 9; with as little as 0·1 % glucose there is some delay in the inactivation.

Many attempts have been made to test the protective effect of salts which have eutectic temperatures above the temperature of freezing, and which will precipitate the virus. Crystals with the usual dodecahedral form can be made by precipitating the virus at 0° with $FeSO_4$, $MgSO_4$, $ZnSO_4$, $MnSO_4$ and $Na_2S_2O_3$; the eutectic temperatures of these salts are −1·8, −3·9, −6·5, −10·5 and −11·0° respectively. In some experiments these salts have been found to exert a greater protective effect than salts such as $CuSO_4$, KNO_3, KCl and $BaCl_2$ that do not precipitate the virus at 0°, but we have not been able so to define the conditions that consistent results can be obtained.

Table 8. *Effect of salts with different eutectic temperatures on purified virus preparations*

Salt	Eutectic temp.	Conc. at the eutectic of salt/100 g. of water (g.)	g. of salt/100 g. of water in the solution frozen	Amount of material insoluble after freezing at (arbitrary units, see p. 73)		Activity after thawing from	
				−10°	−20°	−10°	−20°
KNO_3	− 2·9°	12·2	4·9	3	3	−	−
KCl	−11·1°	24·0	9·6	1	.	+	.
NH_4Cl	−15·8°	22·9	9·2	0	3	+	−
NaCl	−21·2°	28·9	11·5	0	.	+	.
$MgCl_2$	−33·6°	27·5	11·0	0	0	+	+

Each test was carried out on a solution containing 40% of the eutectic concentration of the salt, 1·4 g. virus/l. and *M*/10 phthalate buffer at *p*H 4·4. In all cases the duration of freezing was 68 hr.

Table 9. *The protective effect of different materials on purified virus preparations*

In all these experiments 0·1 ml. lots of a 10 g./l. solution of virus in *M*/25 phthalate buffer at *p*H 4·0 were kept frozen at −10°. In addition the fluids contained the following substances.

Time of freezing	Salt added	Amount of material insoluble after thawing (arbitrary units, see p. 73)	Serological end-point	Av. no. of lesions per leaf at	
				10^{-5}	10^{-6}
1 min.	No addition	1	1:320,000	63	8
	1·6% KNO_3	0	1:320,000	29	7
	0·27% NaCl	0	1:320,000	30	12
	1·5% glucose	0	1:320,000	49	8
1 hr.	No addition	3	No ppt.	7	1
	1·6% KNO_3	2	1: 40,000	20	1
	0·27% NaCl	0	1:320,000	20	5
	1·5% glucose	0	1:320,000	23	3
24 hr.	No addition	3	No ppt.	0	0
	1·6% KNO_3	3	No ppt.	0	0
	0·27% NaCl	1	1: 80,000	3	0
	1·5% glucose	0	1:320,000	18	5
0	No addition	0	1:320,000	48	22

Effect of freezing infective sap

In infected leaves and in expressed sap, bushy stunt virus is apparently unaffected by freezing for days or even weeks. The results that have been described above give ample reason for this. Even the most highly infective sap has only a quarter of the virus concentration that is necessary for inactivation at the isoelectric point. Tomato sap has never been found with a *p*H lower than 5·6, and the salts and sugars in the sap supplement these other protective effects. After dialysis, infective sap is still not inactivated if it is frozen at its own *p*H, but it is inactivated if acidified before freezing; this is shown in Table 10. The sap from minced tomato leaves was frozen, centrifuged and dialysed for 48 hr. against frequently changed distilled water. After centrifuging again, it contained 1·8 g. solids/l. and was at *p*H 6. Samples were adjusted to *p*H 3, 4 and 5 with *N*/10 H_2SO_4, and 2 ml. lots were frozen at −10° for 14 hr. They were all centrifuged after thawing, although there was no precipitate in the one at *p*H 5, and tested. It is clear that with this dilute virus solution, inactivation took place only at *p*H 3; the serum precipitation end-point suggests that this dialysed sap contained about 0·02 g. virus/l.

Table 10. *The effect of p*H *on the freezing of infective sap*

*p*H of sap	Serum precipitation end-point		Av. no. of lesions per leaf	
	Frozen	Unfrozen	Frozen	Unfrozen
5	1:8	1:8	64	85
4	1:8	1:8	68	91
3	No ppt.	1:8	6	65

Virus is also protected by the colloidal constituents of sap from inactivation by freezing. The effect is not very definite, but a solution containing 5 g. virus/l. and at *p*H 4·0 was only half inactivated in 18 hr. at −9°, when half of the final volume of solution consisted of dialysed healthy sap prepared as in the preceding experiment. When the proportion of sap colloids was only one-sixth the activity fell to one-eighth, and with less sap than this there was no protection. The colloidal material present in sap prepared in this way is largely polysaccharide,

but, since it also contains 3–4 % of N, a few experiments on protection by proteins have been made. Dialysed preparations of rabbit-serum albumin and pseudoglobulin were used and, as Table 11 shows, they had a substantial protective effect.

Table 11. *The protective effect of proteins on virus exposed to freezing*

Each mixture contained 2 g. of virus/l. dissolved in *M*/20 *p*H 3·5 phthalate buffer solution, the addition specified was also made. All except the control were kept frozen at −10° for 14 hr.

Addition	Amount of material insoluble after thawing (arbitrary units, see p. 73)	Serum precipitation end-point	Av. no. of lesions per leaf 10^{-4}	Av. no. of lesions per leaf 10^{-5}
None	3	0	0	0
1·6 % albumin	2	1 : 80,000	6	7
0·5 % albumin	3	1 : 40,000	7	0
1·6 % pseudo-globulin	1	1 : 320,000	48	11
Unfrozen control	0	1 : 320,000	169	53

Loss of infectivity without loss of serological activity

In general, loss of infectivity in frozen samples of bushy stunt virus is accompanied by a corresponding loss of serological activity and by the production of insoluble materials. However, inactivation by freezing, as by heating, is a complex process, and loss of infectivity is produced by the earliest changes. It is more difficult to make serologically active but non-infective preparations by freezing than by heating, but the results in several of our tables show that some of the frozen samples have suffered a greater loss of infectivity than of serological activity.

As with so many other aspects of this study, conditions cannot be defined which will always give a separation of the two activities on freezing. It is probable that the lack of fine control over the rate of freezing accounts for much of the variability. The most consistent results have been obtained with 0·2 % virus solutions in *M*/10 phthalate or acetate buffer at *p*H 4·25 kept at −10° for 2 hr. In about half the tests under these conditions, preparations have been non-infective although fully active serologically, and no other conditions have been found that give a higher proportion of successes. Inactivation of this type has no effect on the sedimentation constant. When such non-infective preparations were set to crystallize with $(NH_4)_2SO_4$, most of the material crystallized in the form of the usual dodecahedra, but the supernatant fluids showed a slight shimmer when shaken. Such an effect has not been noticed during the crystallization of normal preparations of bushy stunt virus, although it has been during attempts to crystallize serologically active preparations made non-infective by heating in alkaline solution. On long standing in the presence of $(NH_4)_2SO_4$ a few birefringent crystals have separated from virus preparations made non-infective, but not denatured, by freezing.

The fact that freezing can produce this type of inactivation needs to be remembered when virus preparations are being made from frozen leaves or sap, for much of the non-infective virus is indistinguishable, by the criteria we have used, from fully active virus. It is perhaps unlikely that inactivation of this type would occur under the conditions obtained in frozen sap, but if it did, infective and non-infective virus would not be separated by the ordinary purification procedure.

DISCUSSION

So many treatments can cause loss of infectivity without producing any appreciable changes in physical, chemical or serological properties, that attempts to assess the homogeneity of purified virus preparations are likely to give indecisive results until much more sensitive methods of testing for infectivity are discovered. The local lesion method is adequate for comparing the relative activity of different preparations, but it tells little or nothing about the absolute activity. The fact that the same virus preparation can give widely varying numbers of lesions on different plants of the same species, or on different leaves of the same plant, shows that the number of lesions formed in any one test bears no fixed relationship to the quantity of active virus in the inoculum. Even when using the most sensitive leaves, however, some thousands of times the amount of virus has to be applied that would be needed in theory if one virus particle can cause infection. This discrepancy may arise because of the wastage of inoculum in the relatively crude methods of inoculation used, or it may be that large numbers of active virus particles are needed to cause one lesion. The latter possibility could be tested by the method used with vaccinia [Parker, 1938; Haldane, 1939], that is to say, by determining the proportion of inoculations that fail to give any lesions when different amounts of virus are present in the inoculum. An equally probable explanation, however, is that even the best preparations of virus yet made consist largely of virus that has lost infectivity but not serological activity. Although this type of inactivation is a complicating factor in work on the purification of viruses, its occurrence makes viruses particularly suitable material for studying the earliest changes that lead to denaturation. With proteins that have no specific biological activities, changes can only be detected when the physical, chemical or serological properties have been altered measurably.

Ideally, a pure virus preparation would consist of particles all identical in size, constitution and ability

to cause infection, but there is no evidence that this ideal has ever been achieved. Deviations from it can arise in four main ways. First, the purified preparations may still contain material that is either a constituent of the normal host or a product of infection that is inessential for infectivity. The detection of such contaminants should not present serious problems, and it is probable that preparations of some viruses have been made substantially free from them. In this connexion it should be remembered that, although it may be legitimate to regard the more active of two preparations with equal solid content as the more pure, all contaminants do not necessarily reduce infectivity and serological activity [Pirie, 1940]. Secondly, even when all the particles in a virus preparation are fully active, these particles may be of different kinds. Most preparations of tobacco mosaic virus fall into this category, for even cultures derived from single local lesions are often mixtures of virus strains [Jensen, 1936]. Thirdly, during the course of isolation some or all of the virus particles may have become modified so that, although the final product consists solely of virus particles or of particles derived from them, these may vary in activity. The detection of inactive contaminants arising in this manner presents serious problems only when the modification is slight. Finally, all the virus particles in an infected leaf may not be equally infective although inseparable by the available methods. Particles less active than the ideal may be stages in the production of fully active virus or they may be particles that have suffered inactivation. The first alternative would offer a ready explanation for Spencer's [1941] observation that, weight for weight, tobacco mosaic virus from recently infected plants is less infective than virus from plants infected for longer periods. The second might also be expected as it is analogous to the type of inactivation that occurs in sap extracted from plants suffering from bushy stunt or tobacco necrosis.

In the past the thermal inactivation point has been widely used as a property for characterizing individual viruses, and with most viruses different workers have usually agreed within narrow limits about the temperature at which 10 min. heating causes loss of infectivity in expressed sap. The data on bushy stunt and tobacco necrosis viruses, however, have been conflicting. Provided the *p*H and other conditions of heating are controlled, reproducible results are readily obtained with potato virus '*X*' and tobacco mosaic virus. But with the same preparation of bushy stunt virus, the thermal inactivation point may vary over 15° in successive tests. These differences seem to arise because of the different coefficients of thermal inactivation for the different types of viruses. Loss of infectivity with virus '*X*' and tobacco mosaic virus is closely linked with denaturation and coagulation of the protein and it has a large temperature coefficient, so that the precise temperature to which the preparation is heated is of the greatest importance in determining loss of infectivity. With bushy stunt virus, on the other hand, loss of infectivity is not closely linked with denaturation and the former has a small temperature coefficient. Thus the exact temperature of heating is of little more importance in determining the apparent point of inactivation than other factors such as concentration of inoculum and susceptibility of the test plants. With such viruses, determinations of the thermal inactivation point are obviously of reduced value as a diagnostic character. Measurements of the rate of inactivation at different temperatures, however, may become of value in differentiating between those viruses with widely different coefficients of thermal inactivation. In our earlier paper on bushy stunt virus, we stressed inactivation by freezing as a property that sharply differentiated this from the other viruses we had studied, for none of the others had been affected by freezing. However, we did not then realize that the inactivation of bushy stunt virus was so dependent on the precise conditions of freezing, and other viruses may be similarly inactivated if the freezing is done under more critical conditions.

SUMMARY

Tomato bushy stunt virus loses its infectivity when heated insufficiently to cause denaturation and loss of serological activity. The temperature coefficient for loss of infectivity is small and for loss of serological activity is large. The amount of heating needed for denaturation varies with the *p*H.

No differences have been found between the chemical and physical properties of non-infective, but serologically active, material and those of fully active preparations.

The rate of inactivation by freezing is increased by increases in the concentration of the virus, in the duration of freezing and in the acidity of the fluid. The virus is protected from inactivation by salts and some other substances. The efficiency of different salts depends on the salt : ice : water eutectic temperature.

In general, loss of infectivity is accompanied by the separation of a precipitate and loss of serological activity, but in some conditions freezing destroys infectivity without altering serological activity.

REFERENCES

Bawden, F. C. [1941]. *Brit. J. exp. Path.* **22**, 59.
—— & Pirie, N. W. [1937]. *Proc. roy. Soc.* B, **123**, 274.
—— —— [1938*a*]. *Brit. J. exp. Path.* **19**, 251.
—— —— [1938*b*]. *Nature, Lond.*, **142**, 842.
—— —— [1938*c*]. *Brit. J. exp. Path.* **19**, 264.
—— —— [1943]. *Biochem. J.* **37**, 66.
Blanchard, K. C. [1940]. *Symp. quant. Biol.* **8**, 1.
Haldane, J. B. S. [1939]. *J. Hyg., Camb.*, **39**, 289.
Holzapfel, L. & Nord, F. F. [1938*a*]. *Naturwissenschaften*, **26**, 283.
—— —— [1938*b*]. *Ber. dtsch. chem. Ges.* **71**, 1217.
Jensen, J. H. [1936]. *Phytopathology*, **26**, 266.
Jung, E. [1931]. *Kolloidchem. Beih.* **32**, 320.
Kausche, G. A. & Holzapfel, L. [1940]. *Naturwissenschaften*, **28**, 62.
Kistler, S. S. [1936]. *J. Amer. chem. Soc.* **58**, 901.
Luyet, B. J. [1937]. *Biodynamica*, **29**, 1.
—— & Thoennes, G. [1938]. *Science*, **88**, 284.
McFarlane, A. S. & Kekwick, R. A. [1938]. *Biochem. J.* **32**, 1607.
Moran, T. [1926]. *Proc. roy. Soc.* A, **112**, 30.
Nord, F. F. [1933]. *Ergebn. Enzymforsch.* **2**, 23.
—— & Lang, F. E. M. [1935]. *Nature, Lond.*, **135**, 1001.
Parker, R. F. [1938]. *J. exp. Med.* **67**, 725.
Pirie, N. W. [1940]. *Biol. Rev.* **15**, 377.
Price, W. C. [1938]. *Amer. J. Bot.* **25**, 603.
—— [1940]. *Arch. Virusforsch.* **1**, 373.
Spencer, E. L. [1941]. *Science*, **94**, 96.
Stanley, W. M. [1940]. *J. biol. Chem.* **135**, 437.

Note added 19 Nov. 1942. Since this paper was written a comprehensive review (Breedis, C. [1942], *J. exp. Med.* **76**, 221) on the effects of freezing on a variety of biological materials has appeared. It also contains experiments showing the importance of controlling the rate of freezing in the destruction of leukaemia cells.

Addendum: Examination of Bushy Stunt Virus in the Ultracentrifuge

By A. G. OGSTON, *Department of Biochemistry, Oxford*
with the technical assistance of W. Weinstein

Nine samples of bushy stunt virus were examined in a Svedberg oil-turbine ultracentrifuge by the 'diagonal schlieren' method [Philpot, 1938]. The speed of rotation was 300–310 rev./sec.; the solutions, of concentration 0·35 or 0·25 % in 0·1 *M* NaCl with 0·01 *M* buffer (acetate *p*H 4·7 or phosphate *p*H 6·8), were contained in a 12 mm. cell.

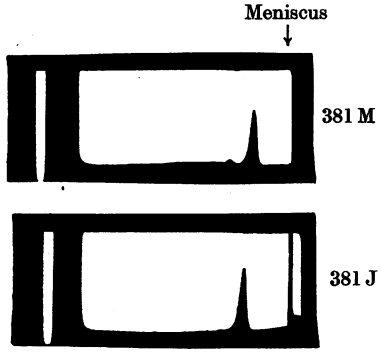

Fig. 1.

The results and details are given in Table 1 and specimen schlieren diagrams in Fig. 1.

All the preparations (except 381 M which contains a small amount of a more rapidly sedimenting component) appeared to be homogeneous. In three cases the fraction of the total refracting protein which appears in the boundary was measured by the method of Philpot [1939]; these figures are given in Table 1; but because of the sharpness of the boundaries, and the smallness of the areas measured, the figures are not very accurate; they indicate that the samples are largely homogeneous.

The sedimentation constants obtained here agree with the mean value obtained in five other laboratories on Lauffer & Stanley's [1940] virus, including the value of 133 obtained by McFarlane. It differs significantly from the value of 146 which McFarlane & Kekwick [1938] obtained on an earlier virus preparation by Bawden & Pirie [1943], but I am asked by these authors to state that this value is probably too high because of irregularities of temperature in the rotor of their Svedberg equilibrium centrifuge, which they had not suspected at the time. Recently the same virus preparation (bushy stunt·515J of Bawden & Pirie) has been examined in both laboratories (0·25 % in acetate buffer *p*H 4·0 of ionic strength 0·02) and the mean of three values (Ogston) was 129, while the mean of four values (McFarlane & Kekwick) was 133.

Table 1. *Examination of bushy stunt virus in the ultracentrifuge*

Run	Index no.	Type of product	Conc. %	pH	$S^{20°}_{W} \times 10^{-13}$	Fraction in boundary
270	381J	Unheated	0·25	4·7	135·0	0·87
269	368G	Unheated	0·25	4·7	130·3	—
274	381K	Unheated	0·25	4·7	133·8	—
275	431B	Heated after purification	0·25	4·7	130·1	—
273	381M	Heated after purification	0·25	4·7	133·4	—
					225	*ca.* 0·05
266	363G	Prep. from heated sap	0·25	4·7	130·8	0·93
267	363G	Prep. from heated sap	0·25	6·8	135·9	—
268	350B	Material inactivated by lying in sap	0·35	4·7	130·2	0·89
297	531A	Inactivated by freezing	0·25	4·7	132·7	—
298	531B	Inactivated by freezing	0·25	4·7	131·1	—

REFERENCES

Bawden, F. C. & Pirie, N. W. [1943]. *Biochem. J.* **37**, 70.

Lauffer, M. A. & Stanley, W. M. [1940]. *J. biol. Chem.* **135**, 463.

McFarlane, A. S. & Kekwick, R. A. [1938]. *Biochem. J.* **32**, 1607.

Philpot, J. St L. [1938]. *Nature, Lond.*, **141**, 283.

—— [1939]. *Biochem. J.* **33**, 1725.

Partition Chromatography in the Study of Protein Constituents

By A. H. GORDON, A. J. P. MARTIN and R. L. M. SYNGE, *Wool Industries Research Association, Torridon, Headingley, Leeds*

(*Received 13 October 1942*)

In the present paper we report new applications and developments of partition chromatography* [Martin & Synge, 1941*b*] in the study of amino-acids and peptides. We have further developed the technique, aiming first at the isolation and identification of protein constituents, both amino-acids and peptides, and secondly, at a reliable micro-analytical procedure for the determination of as many different amino-acids as possible in the same sample of a complete hydrolysate of a protein or peptide.

Here we describe the more technical aspects of the work and give a detailed account of the micro-method for monoamino-acids at its present stage of development, together with the results obtained with it on wool and gelatin hydrolysates. The two following papers illustrate further the application of our new methods to particular problems in protein chemistry. Throughout the present work we have employed, as previously, the acetyl derivatives of the amino-acids and peptides.

* We employ the term 'partition chromatography' at the suggestion of Dr E. Lester Smith, to distinguish it from the classical adsorption chromatography. Our earlier term 'liquid-liquid chromatography' was liable to confusion with the fractional elution procedure sometimes called 'liquid chromatography'.

EXPERIMENTAL

Preparation of silica gel for chromatography

Adsorption by the silica of the substances undergoing analysis could seriously alter their behaviour from that to be expected were their partition between the two liquid phases the only determining factor. There must be an adequate concentration of anti-adsorbent substance in the solvent employed [Martin & Synge, 1941*b*]. Silica gel prepared in different ways has very different adsorption properties, and the more adsorbent gels may require unsuitably high concentrations of alcohol before they are useful. Adsorption by the silica may manifest itself as 'tailing' of the solutes undergoing analysis, and results in imperfect separation. This is the behaviour to be expected for adsorption of the 'Freundlich' type, where, for low concentrations, the adsorption is relatively much greater than for high ones. 'Ageing' of the freshly precipitated gel under dilute acid before drying it is essential for the production of a suitable colour with methyl orange (MO), but if ageing is continued for much longer than prescribed, a serious increase in adsorbing power may result. Deviations from the precipitation procedure given below (order of addi-