Rothamsted Repository Download

A - Papers appearing in refereed journals

Bawden, F. C. and Pirie, N. W. 1940. The effects of alkali and some simple organic substances on three plant viruses. *Biochemical Journal.* 34 (8-9), pp. 1278-1292.

The publisher's version can be accessed at:

- https://dx.doi.org/10.1042/bj0341278

The output can be accessed at: https://repository.rothamsted.ac.uk/item/8w407.

© 1 September 1940, Portland Press Ltd.

151. THE EFFECTS OF ALKALI AND SOME SIMPLE ORGANIC SUBSTANCES ON THREE PLANT VIRUSES

By F. C. BAWDEN

Rothamsted Experimental Station, Harpenden, Herts

AND N. W. PIRIE

Biochemical Laboratory, Cambridge

INTRODUCTION

AT the end of last century it was widely recognized that many substances, notably choline [Mauthner, 1874], sodium myronate and the mustard oils, could dissolve otherwise insoluble proteins, and this property was applied clinically by some workers. Hebra [1892] and Mendel [1905] recommended thiosinamine (sold dissolved in sodium salicylate under the name "Fibrolysin") for the softening and ultimate removal of scar- and fibrotic-tissue, and Fraenkel [1915] also recommended choline for this purpose. The clinical use of urea, to which we have previously referred [Bawden & Pirie, 1940], seems to have been developed independently of these observations. Spiro [1900] quoted many of the scattered observations of the early workers and described the solvent action, demonstrated either by dissolving a coagulum or by raising the temperature of heat coagulation, of urea, choline, piperidine, pyridine, urethane, formamide and mustard oils. He concluded that some of these acted simply as bases but that others formed compounds with the proteins. He [1900; 1904] also observed that some of these agents were protein precipitants when dilute although they were solvents when concentrated. A similar effect was noticed by von Furth [1896] during a study of the coagulation of muscle and serum proteins by salicylate and several alkaloids and it has been observed and commented on by many workers since. Neuberg [1916] studied the effects of salts of 43 organic acids, mainly aromatic, on the solubilities of casein and several other substances normally difficult to dissolve, and he found that benzoates, hippurates and many substituted benzoates increased solubility greatly. Recent papers have tended to be less extensive, concentrating on the action of one substance or on a group of closely related ones. Stoeltzner [1925], Hopkins [1930], Pauli & Weiss [1931], and Edsall & Mehl [1940], however, have compared the actions of a wide range of substances on a small number of test objects.

Svedberg and his colleagues studied protein solutions containing protamines, arginine or lysine, in the ultracentrifuge, and found that the proteins were often dissociated into apparently homogeneous particles of smaller molecular weights. This was a valuable confirmation and extension of the osmotic pressure measurements made on proteins dissolved in urea and acetamide. Details of the experiments have not been published [cf. Svedberg & Pedersen, 1940], but from the published summaries [Svedberg, 1937; Lundgren, 1938; Pedersen, 1938] it is clear that there was considerable specificity of action. For example, in the

presence of ammonium chloride arginine dissociated serum albumin but not *Helix* haemocyanin, whereas lysine dissociated the haemocyanin but not the albumin, and neither acted without the ammonium chloride.

In the experiments described in this paper purified preparations of the three plant viruses, tobacco mosaic, tomato bushy stunt and potato "X", were used. The methods of preparation and of testing were the same as in the experiments with urea [Bawden & Pirie, 1940]. For a number of reasons the results obtained with these purified virus preparations may differ from those that might have been obtained with crude preparations. First, the viruses may have undergone physical or chemical changes during purification. Secondly, the large amounts of normal plant proteins and other materials in infective saps might remove the agents added and so protect the viruses from their effects. Thirdly, concentrations of the agents too dilute to affect the viruses directly may produce a coagulum of normal plant protein and still active virus may be adsorbed on to and removed by this.

EXPERIMENTAL

Sodium dodecyl sulphate and alkali

The hydrogen sulphate of dodecyl alcohol (S.D.S.) was used by Bawden & Pirie [1938, 1, 2] to inactivate potato virus "X" and tomato bushy stunt virus and it was found to separate the nucleic acid from the proteins. The work was extended to tobacco mosaic virus by Sreenivasaya & Pirie [1938], who made a more detailed study of the nucleic acid and the water-soluble, phosphorus-free protein produced by its action on this virus. Of the three viruses, potato virus "X" is the most susceptible to the action of S.D.S. and tomato bushy stunt virus the least. Even potato virus "X", however, is more resistant than other proteins that have been tested. S.D.S. has little effect on this virus at dilutions greater than 0·3 % in neutral solution; although changes in haemoglobin [Anson, 1939], phyllochlorin [Smith, 1940] and cytochrome *c* [Keilin & Hartree, 1940] have been brought about rapidly at 10 times this dilution. Analogous changes are caused by some other surface active substances, e.g. soaps and bile salts, and these have been discussed in the papers quoted.

Sreenivasaya & Pirie [1938] found that tobacco mosaic virus was almost inactive after 24 hr. in 1 % S.D.S. at 37° and pH 8. If either the temperature or the pH is raised the inactivation proceeds more rapidly and more dilute S.D.S. can be used, but the effect is complicated by the inactivation caused by the heating or alkali alone. At either room temperature or 37° the products of alkaline inactivation of tobacco mosaic virus remain soluble in the presence of small amounts of salts, although they are readily coagulated by strong salt solutions. At 55° or higher, however, the protein precipitates as it is liberated from combination with the nucleic acid unless the solution is more alkaline than pH 11. In tests in which tobacco mosaic virus at pH 10-14 was heated for varying periods at 55°, the falls in infectivity and serological activity were found to be proportional to the weight of protein precipitated, and this weight when plotted against the log of time gave a straight line. After 160 min. the preparation was completely inactivated, and 87 % of the starting material was precipitated as a carbohydrate-free protein, a figure agreeing well with the expected content of protein free from nucleic acid. As our results on the increase in the rate of alkaline inactivation of tobacco mosaic virus by heat, which may equally well be regarded as a reduction in the thermal inactivation point by increasing pH, agree in all essentials with those of Lauffer & Price [1940] they need not be given in detail. This effect is not confined to tobacco mosaic virus,

for at 55° both potato virus "X" and tomato bushy stunt virus are rapidly inactivated at pH values that are without appreciable effect at room temperature.

Table 1. *The effect of sodium dodecyl sulphate on tobacco mosaic virus*

| | | | Average no. of lesions per leaf at | |
Sample	Concentration of s.d.s.	Serological titre	10^{-4}	10^{-5}
A	0	1/1,280,000	6	1
B	0·043 %	1/640,000	2	1
C	0·073 %	1/160,000	0	0
D	0·143 %	1/10,000	0	0
E	0·073 % neutralized	1/1,280,000	104	47
F	0 control	1/2,560,000	205	95

Samples A, B, C, and D were 0·3 % solutions of tobacco mosaic virus in M/15 glycine : NaCl buffer at pH 9·3 exposed for 1 hr. at 55° to the concentration of s.d.s. stated. They were then taken to pH 6 with HCl and phosphate buffer and diluted for testing. In E the virus was added to a neutralized and diluted buffer mixture containing as much s.d.s. as C, and in F virus was added to neutralized buffer only.

Table 1 shows the effect of s.d.s. on tobacco mosaic virus at pH 9·3 and 55°. In the absence of s.d.s. there is a definite fall in the infectivity of the preparation that is not paralleled by a similar fall in serological titre. A similar dissociation of these two properties with tobacco mosaic virus can be produced by oxidizing agents, nitrous acid, formaldehyde and irradiation with X-rays or ultra-violet [Bawden & Pirie, 1937; Stanley, 1936], and with tomato bushy stunt virus by alkali [Bawden & Pirie, 1938, 2]. The conditions of pH and temperature within which there is complete, or almost complete, loss of infectivity with tobacco mosaic virus without great loss of serological activity are narrowly circumscribed, and it is seldom that such a definite difference as that between samples A and E in Table 1 is obtained. Samples which lose their infectivity but not their serological activity in this manner retain their ability to show anisotropy of flow. With slightly more severe treatment, however, secondary changes rapidly follow those that lead only to a loss of infectivity, and the preparations lose both their serological activity and anisotropy of flow.

It is clear that the action of alkali on tobacco mosaic virus is complex. Eriksson-Quensel & Svedberg [1936] and Wyckoff [1937] found that after exposure to alkali between pH 9 and 11 tobacco mosaic virus was split into a number of products of smaller molecular weight and that the extent of the disintegration depended on the pH. These workers made no measurements on the activities of their preparations after these treatments, but Best [1936] found that both purified and crude preparations were 50 % inactivated by 12 hr. exposure to pH 8·2 at 17°. The purified preparations that we have used have been much more resistant to alkali than this. Table 2 shows the results of one of our experiments. There was no difference between the control and a sample that had been held at pH 8·58 for 24 hr. at 20°, and only above pH 10 was there great inactivation. The treatment at pH 10·5 again shows the dissociation of infectivity from serological activity. The treatment at pH 9·3 shows an effect which we have frequently obtained in these alkali tests, an apparent increase in the infectivity by gentle treatment. This activation can be regarded as a partial reversal of the fall in infectivity that is produced by rigorous chemical purification of the virus. This is believed to be caused by the linear aggregation of

Table 2. *The effect of alkali on tobacco mosaic virus*

| | | | Average no. of lesions per leaf at | |
| | | Serological | | |
pH	Time	titre	10^{-4}	10^{-5}
11	24 hr.	1/40,000	0	0
10·5	24 hr.	1/3,000,000	12	2
9·3	24 hr.	1/4,000,000	106	21
8·5	24 hr.	1/4,000,000	74	9
8·5	5 min.	1/4,000,000	65	11
	Control	1/4,000,000	73	10

In each sample 0·1 ml. of 5·3 % tobacco mosaic virus solution was added to 1 ml. of $M/10$ glycine: NaCl buffer at the pH stated. 0·08 and 0·06 ml. of $N/10$ NaOH were added to the first and second, for interpolation on the titration curve of tobacco mosaic virus shows that these amounts are necessary to bring 5·3 mg. of virus to pH 11 and pH 10·5 respectively. After the time stated, each was neutralized with acetic acid and diluted for testing. In the control, virus was added to 1 ml. of pH 8·5 buffer previously neutralized with acetic acid.

virus particles, and the simplest explanation of the reactivation is that the alkali disaggregates the long rods. Thus at least three successive effects of alkali on tobacco mosaic virus can be detected. First, there is a disaggregation of the purified material, secondly, a change within the particles that renders them non-infective without destroying their structure or serological properties and thirdly, a disruption of the particles that leads to a loss of all characteristic properties. The course of the third stage is also influenced by the temperature, for at low temperatures the products of denaturation remain soluble, whereas at high temperatures they coagulate.

The action of s.d.s. in the presence of alkali differs from the action of alkali alone. For example, in the presence of sufficient s.d.s. to cause appreciable inactivation of tobacco mosaic virus there is no precipitate of denatured protein such as would be produced by the action of alkali and temperature alone. Thus, in the treatments described in Table 1, although A contained a precipitate, B and C were only opalescent and D was perfectly clear. In attempts to gain further information on the antigenic constitution of tobacco mosaic virus, rabbits were injected with the phosphorus-free protein obtained by treating the virus with s.d.s., but the serum failed to react with either intact virus or that treated with s.d.s.

Tomato bushy stunt virus is even less readily attacked by s.d.s. than tobacco mosaic virus, and in neutral solution it has never been completely inactivated in any of our experiments. This may be because an equilibrium is established, but we have no definite information on this. In solutions containing 4·4 % s.d.s. and 2·6 % bushy stunt virus and 3·5 % $Na_2HPO_4,12H_2O$ precipitates separate almost immediately, the precipitates appearing amorphous under the microscope. Like the similar, though shimmering, precipitates that separate with tobacco mosaic virus, these dissolve in about 30 min. at 37°. In these conditions a second precipitate, apparently a complex of s.d.s. and modified protein, separates after a few hours; this precipitate, like that given by the unchanged virus with ammonium sulphate, dissolves when the fluid is cooled. After 20 hr. at 37°, a dialysed sample still contains about 10 % of unchanged virus. This mixture can be partially separated either by centrifuging at 16,000 r.p.m. or by ammonium sulphate precipitation, for the inactive products formed by the action are not sedimented by a few hours' centrifuging at this speed and they are more easily precipitated by ammonium sulphate than the virus, especially at 0°. The inactive

protein that can be separated by the addition of ammonium sulphate to slightly alkaline solutions is almost free from phosphorus and carbohydrate, and nucleic acid can be precipitated from the supernatant fluids by the addition of acid.

With tomato bushy stunt virus it is much easier to destroy infectivity without destroying serological activity by means of alkali than it is with tobacco mosaic virus. We have previously described [Bawden & Pirie, 1938, 2] this effect when bushy stunt virus is exposed to pH 11 at 18°, but at higher temperatures the effect is more definite and can be produced in less alkaline solutions. In one experiment exposure for 30 min. at 55° at pH 9 and 9·7 in $M/10$ glycine buffer gave no change in the serological titre, although at a dilution of 10^{-4} g. of protein per ml. the samples gave 3 and 0 lesions respectively, whereas the control mixed with neutralized buffer at 55° gave 720 lesions. From preparations treated in this manner rhombic dodecahedral crystals can be obtained by slow precipitation with ammonium sulphate that are apparently identical with those formed by fully active virus. This non-infective crystalline material is indistinguishable, by the chemical, physical and serological tests that we have applied, from the virus isolated directly from infective sap, but careful solubility measurements have not yet been made. The existence of a protein that is not infective although it closely resembles the active virus in its properties suggests the possibility that such a material may often be a contaminant of purified virus preparations as normally prepared. This is especially likely as a similar type of inactivation seems to proceed in crude infective sap, although here, as the pH is usually around 5·5, the effect cannot be attributed to alkaline inactivation. Smith [1937] observed that infective sap from bushy stunt plants rapidly lost its infectivity and was inactive within a month. We have confirmed this observation but have found in striking contrast that purified preparations do not lose their infectivity in this manner. After more than a year at room temperature the infectivity of purified preparations of this virus has not been found to be significantly reduced. Samples of infective sap have been tested for serological activity and infectivity over periods of time, and although in all the infectivity has been found to fall off rapidly the serological activity has remained constant. Samples of infective sap kept over a year at room temperature and which had been non-infective for a year were still found to be serologically active, and from them we isolated a non-infective crystalline nucleoprotein by the methods used for normal virus isolation. This material was found in the same quantities and gave the same serological titre as fully active virus preparations. The possibility that preparations may consist of mixtures of infective and non-infective nucleoprotein may explain the fact that different preparations give remarkably constant serological titres but differ in their infectivities.

Bushy stunt virus preparations lose their activity in alkaline solutions more rapidly in the presence of s.d.s. and inactivation occurs at lower pH values. The action is also not restricted to one that merely destroys infectivity, for the nucleic acid is split off from the non-infective protein and the preparation loses its serological activity. In one test in which a 1 % solution of the virus was exposed for 160 min. to 2·6 % s.d.s. at pH 9 and 18°, the preparation lost its infectivity but could be split into various fractions after dialysing for a week at 0°. The addition of 1/8 vol. of saturated ammonium sulphate solution precipitated a nucleic acid-free protein, which represented 75 % of the original weight of virus, was amorphous and did not react with virus antiserum. With more ammonium sulphate there was slow crystallization at 0° of a non-infective, though serologically active, nucleoprotein similar to that already described. The yield of this was 12 % of the starting material.

The absorption spectrum of tomato bushy stunt virus has a pronounced maximum at 260 mμ. After partial inactivation by s.d.s. the curve has the same shape but it lies below the curve for fully active virus. Thus a 2 cm. layer of a 0·016 % solution of the virus transmitted 1/100 of the incident light at 260 mμ ($\log_{10} I_0/I = 2$), whereas after treatment with s.d.s. so that half of the virus was destroyed the transmission was doubled at 260 mμ ($\log_{10} I_0/I = 1·7$). We have described a similar result during the inactivation of potato virus "X" and tobacco mosaic virus by urea, during which the nucleic acids are separated from the proteins. In the inactivation of bushy stunt virus by urea, on the other hand, the nucleic acid is not liberated, and there is no such decrease in opacity.

Urethane

The pharmacological action of the urethanes led naturally to their use in the study of enzyme systems, and Warburg & Wiesel [1912] pointed out that there was a correlation between the ability of a narcotic to give a precipitate with yeast maceration juice and its inhibitory action on the respiration of various tissues. Meyerhof [1918] confirmed these observations, and found that the presence of salt was necessary for the formation of a precipitate by urethanes in protein solutions. Hopkins [1930] measured the rate of denaturation of egg albumin in urethane and the slower rate of denaturation of sheep serum proteins, and he commented on the fact that the solvent powers of urethane were less than those of urea. On the other hand, Jirgensons [1936] found that 25 % methylurethane impeded the precipitation of casein by salts.

In neutral solution strong ethylurethane rapidly denatures tobacco mosaic virus and is a solvent for the denatured products even in the presence of salts. At pH 7 the anisotropy of flow of virus solutions disappears after a few minutes in 28–40 % solutions of urethane and the solutions become perfectly clear, although on dilution a precipitate develops if traces of salt are present. With more dilute urethane the anisotropy of flow does not quite disappear, the loss of infectivity and serological activity is only partial and the inactivated virus precipitates although it can readily be dissolved by the addition of more urethane. The rate of inactivation of tobacco mosaic virus by urea is greatly increased by cooling below 20°, but inactivation by urethane is not. After 48 hr. at 0° a 1·5 M solution of urethane in $M/30$ phosphate buffer of pH 7 containing 0·6 g. of virus per litre gave a serological titre of 1/640,000 and an average number of lesions per leaf of 68 and 5 at dilutions of 10^{-4} and 10^{-5} respectively. After the same time at 0° the preparation gave the same titre and the lesions were 45 and 5, whereas the control gave a titre of 1/1,280,000 and 86 and 19 lesions per leaf.

Table 3. *The effect of urethane on potato virus "X"*

Concentration of urethane M	Serological titre	Average no. of lesions per leaf at	
		10^{-4}	10^{-5}
0·5	1/1,280,000	171	52
1·0	1/640,000	137	41
2·26	No ppt. at 1/10,000	0	0
Control	1/1,280,000	193	64

In each sample a 0·08 % solution of potato virus "X" in $M/50$ pH 7 phosphate buffer was exposed for 24 hr. at 18° to the concentration of urethane stated. They were then diluted with 3 vol. of water and diluted further for testing after 36 hr. For the control an amount of urethane equal to that in the M sample was added immediately before diluting and testing.

The solubility of urethane at $0°$ is too small to permit experiments under conditions in which inactivation is more complete.

Tomato bushy stunt virus is much more resistant to urethane than is tobacco mosaic virus; 22 hr. exposure at $23°$ altered neither the serological titre nor the infectivity of a $0·14\%$ virus solution in $0·023M$ phosphate buffer at pH 7·8. Potato virus "X", however, is more susceptible and Table 3 shows the inactivation of this virus by urethane. The denatured virus was not held in solution by $2·26M$ urethane and at that concentration the anisotropy of flow disappeared almost immediately and a precipitate separated. At the lower concentrations there was no precipitation and only a reduction in the anisotropy of flow.

Guanidine

Guanidine, like many of the other agents used in these experiments, affects proteins in a number of different ways. Petrumkin & Petrumkin [1927; 1928] found that gelatin and a mixture of denatured proteins from brain combined with guanidine in alkaline solution, and Grynberg [1933] measured the amount of combination under various conditions of pH, salt and guanidine concentration, with casein, gelatin, egg albumin and a globulin. Svedberg [1937] studied the dispersive action of guanidine on haemocyanin by means of the ultracentrifuge, and its action on myosin was studied by Edsall & Mehl [1940] who followed the loss of anisotropy of flow and viscosity. Denaturation with the production of —SH groups was found with egg albumin [Greenstein, 1938], excelsin, edestin and globin [Greenstein, 1939], tobacco mosaic virus [Stanley & Lauffer, 1939] and with myosin [Greenstein & Edsall, 1940]. Where comparative tests have been made guanidine has been found to denature at lower concentrations than other agents such as urea, and to produce a larger number of —SH groups. Greenstein [1938; 1939] found with both urea and guanidine that doubling the concentration, within the critical range, more than doubles the amount of —SH produced. This result parallels the well-known effects of variations in the concentration of disinfectants and agrees with the effects of variations in the concentration of the various virus-inactivating agents described in this paper.

Neutral solutions of guanidine hydrochloride, if more concentrated than $0·2M$, precipitate tobacco mosaic virus from solution. The precipitate can be washed with guanidine solution, but readily dissolves in water. Like the precipitate produced when clupein is added [Bawden & Pirie, 1937], it has a fibrous structure, but unlike the clupein precipitate it is not dissolved by the addition of small amounts of salt. There is no loss of activity during this precipitation provided that solutions less than M are used, but in more concentrated solutions there is some denaturation and then the precipitate produced is amorphous. In guanidine solutions more concentrated than $2·5M$ the active virus does not precipitate, but there is rapid inactivation and a precipitate of denatured protein separates. The course of inactivation of tobacco mosaic virus by strong guanidine and its precipitation by dilute guanidine are illustrated in Table 4.

Tomato bushy stunt virus differs from tobacco mosaic virus in its behaviour towards guanidine. It does not precipitate with dilute solutions and concentrated solutions retain the denatured virus in solution. For example, virus exposed to $3·3M$ guanidine did not precipitate on dilution. After inactivation by exposure for 21 hr. at $23°$ to $1·3M$ guanidine, however, a precipitate separated on dilution. It is clear that with these two viruses, as with the proteins that Greenstein has studied, guanidine is effective at lower concentrations than urea.

Table 4. *The effect of guanidine on tobacco mosaic virus*

Treatment	Serological titre	Average no. of lesions per leaf at	
		10^{-4}	10^{-5}
A. 24 hr. in 2·7 M guanidine hydrochloride	No ppt. at 1/10,000	0	0
B. 24 hr. in 2·25 M guanidine hydrochloride	1/80,000	36	4
C. 5 min. in 0·68 M guanidine hydrochloride, then centrifuged:			
(1) Precipitate	1/640,000	200	80
(2) Supernatant	No ppt. at 1/10,000	2	0
D. Control; guanidine hydrochloride added after dilution	1/640,000	209	85
E. Control; no guanidine hydrochloride	1/640,000	228	99

All the tests were made at 17° with 1 ml. samples of a 1·1% solution of tobacco mosaic virus in 0·075 M phosphate buffer at pH 7. To A and B were added 0·4 and 0·2 ml. of 3·38 M guanidine hydrochloride, to C 0·3 ml. of water and 0·2 ml. of guanidine, and to D 2·2 ml. of water and 0·2 ml. of guanidine. After the stated time each was made up to 2·5 ml. with water, and diluted further for testing 36 hr. later.

Pyridine, picoline, lutidine, aniline and nicotine

The action of aqueous pyridine on proteins has been but little studied, although it has long been known to dissolve some [Levites, 1911] and to make gelatin swell even when neutralized [Fischer & Sykes, 1915]. Spiro [1900] observed that dilute pyridine lowered the temperature of coagulation of proteins and changes in haemoglobin [Jirgensons, 1936] and tobacco mosaic virus [Bawden & Pirie, 1937] have been brought about by it.

The neutral pyridine used in our tests was made by diluting 8 ml. of pyridine and 0·5 ml. of N HCl to 25 ml. with water. Neutral picoline was made in the same way. In 3 M pyridine tobacco mosaic virus and potato virus "X" lose their anisotropy of flow almost immediately and give clear solutions from which small, sticky precipitates separate after 24 hr. In more concentrated pyridine the inactivated viruses do not precipitate, while in more dilute pyridine the anisotropy of flow disappears slowly and there is an increase in opalescence leading finally to precipitation. The behaviours of these two viruses in picoline are similar to those in pyridine, but picoline has a slightly greater inactivating power. Tables 5 and 6 illustrate the inactivating effects of pyridine and picoline on tobacco mosaic virus. Potato virus "X" is inactivated under much the same conditions as tobacco mosaic virus, but tomato bushy stunt virus is more resistant. Thus, 20 hr. exposure at 18° to either 2·4 M pyridine or 1·3 M picoline

Table 5. *The effect of pyridine on tobacco mosaic virus*

Concentration of pyridine M	Serological titre	Average no. of lesions per leaf at	
		10^{-4}	10^{-5}
1·33	No ppt. at 1/10,000	0	0
0·625	1/640,000	101	54
Control	1/640,000	118	72

Tobacco mosaic virus solutions containing 0·052% virus and $M/30$ pH 6·95 phosphate buffer were exposed to the concentration of pyridine stated for 24 hr. at 16°. They were then diluted so that the pyridine was 0·312 M and were further diluted for testing 36 hr. later. In the control 0·312 M pyridine was added to the virus immediately before diluting and testing.

Table 6. *The effect of picoline on tobacco mosaic virus*

Concentration of picoline M	Serological titre	Average no. of lesions per leaf at	
		10^{-4}	10^{-5}
0·6	1/80,000	12	3
0·4	1/640,000	66	21
0·2	1/1,280,000	104	51
Control	1/2,560,000	165	69

Each sample contained 1·2 mg. of tobacco mosaic virus and 0·1 ml. of $M/10$ pH 7 phosphate : borate buffer and 0·3 ml. of M picoline in a total volume of either 0·5, 0·75, 1·5 or 2·5 ml. The first three samples were kept 18 hr. at 18° when they were diluted to 2·5 ml., and after 36 hr. they were further diluted for testing. The picoline in the control was added immediately before diluting and testing.

had no effect on the serological titre of bushy stunt virus and only lowered the infectivity slightly. A commercial mixture of lutidines was also tested on tobacco mosaic virus. This was as good a precipitant as pyridine or picoline, i.e. precipitation was complete after 1 hr. in a 4 % solution, but its solubility in water was insufficient to give concentrations great enough to have any solvent action on the denatured virus. Picoline, lutidine and pyridine all separate the nucleic acid from the protein in tobacco mosaic virus.

The protein-precipitating power of aniline has often been commented upon [e.g. Spiro, 1900], and it is therefore surprising that Lauffer [1938] found that exposure for 10 min. to concentrated mixtures of aniline and glycerol had no effect on the infectivity of tobacco mosaic virus. We have been unable to confirm Lauffer's result; 2 vol. of a 2·35 % solution of purified tobacco mosaic virus were added to 98 vol. of a mixture of 48 parts of glycerol and 50 parts of aniline (one of the mixtures used by Lauffer), and samples were withdrawn and diluted for infectivity tests. Dilutions of the virus in water and in the already diluted aniline : glycerol mixture were used as controls. Table 7 shows the results from

Table 7. *The effect of a glycerol : aniline mixture on tobacco mosaic virus*

Time of contact	Average no. of lesions per leaf at	
	10^{-5}	10^{-6}
Diluted immediately	18	10
30 min.	10	6
90 min.	7	4
4 hr.	10	6
Virus added to diluted mixture	83	20
Virus diluted in buffer	84	19

Description in text.

one such experiment. It is apparent that contact of the virus with the glycerol : aniline mixture caused a great immediate reduction in infectivity, but the reduction did not increase greatly as the time of contact was extended. That the effect was one of the mixture on the host plant seems improbable, for at the dilution present in the inoculum there was no fall in infectivity. There is no obvious reason for the difference between Lauffer's results and our own, but the possibility that different strains of tobacco mosaic virus may differ in their resistances to inactivation must be remembered.

The birefringence of tobacco mosaic virus in neutral, salt-free solutions containing up to 20 % of nicotine remains unaffected even after several days and

no precipitate separates. In the presence of salt, however, an opaque, fibrous precipitate separates after a few hours. On gentle shaking this precipitate disappears completely, leaving a fluid indistinguishable in appearance from a normal virus preparation. After a few hours the precipitate again appears and can again be dispersed by shaking; the process can apparently be repeated indefinitely over a period of weeks. A suitable mixture for demonstrating this phenomenon contains 0·4 % of virus, 4 % of NaCl and 7 % of nicotine neutralized with acetic acid, but other mixtures, e.g. those made from neutralized nicotine tartrate, work equally well. Similar, though rather less striking, results are obtained by dissolving tobacco mosaic virus in neutral M arginine hydrochloride. Exposure to this type of precipitating agent does not inactivate the virus, and 2 months' exposure to 15 % nicotine at pH 7 and 18° had no appreciable effect on the infectivity and serological activity. Fukushi [1930] found that 3 % nicotine caused partial inactivation of tobacco mosaic virus in 3 days, but as he was using crude infective sap there is no necessary contradiction between his results and ours.

There are two analogies for this type of reversible precipitation. The first is the birefringent liquid layer that separates from concentrated virus preparations [Bawden & Pirie, 1937], for by vigorous shaking this can be resuspended in the form of tactoids that are too small to be seen and have a refractive index too close to that of the surrounding medium to give a visible opacity. Best [1937] described a precipitate settling from an old sample of clarified, infective sap that may well be identical with the nicotine precipitate. This was also dispersed on shaking and reformed on standing undisturbed. In addition to the physico-chemical interest attached to precipitates of this type, their formation, especially in fluids with compositions similar to plant sap, may throw some light on the mechanism of formation of the characteristic inclusion bodies found in plants infected with certain viruses. The other systems that have been considered as models for this phenomenon [Bawden & Sheffield, 1939] bear less resemblance to normal cell contents than those described here.

Phenol, salicylic acid and benzoic acid

Runge [1834], the discoverer of phenol, found that it was a protein coagulant, and its power of inhibiting enzymes was stressed by Plugge [1872], who was also one of the first to use it as a disinfectant. Buchner & Hoffmann [1907] and Duchacek [1909] correlated this inhibitory power with the precipitation of protein. Innumerable studies with bacteria and animal viruses have shown that 5–10 % phenol is usually lethal. Henderson [1933] found that the stability of tobacco ringspot virus in crude sap was increased by the addition of 0·25 % phenol, and Stanley [1935] found that 1 % phenol had little effect on tobacco mosaic virus. Bawden [1935] found that 2 % phenol acting for 48 hr. at 1° had no effect on potato virus " X ", whereas 3 % destroyed infectivity and either greatly reduced or destroyed serological activity.

In concentrated solutions phenol is usually a protein solvent, but the molecular weights found for proteins dissolved in this manner have been so low [Troensgaard & Schmidt, 1924; Cohn & Conant, 1926] that it is probable that they were dissociated. Measurements in the ultracentrifuge appear to confirm this dissociation [Lundgren, 1938]. The effects of salicylates appear to be similar to those of phenol. Dilute solutions precipitate proteins [von Furth, 1896] whereas strong solutions either dissolve proteins [Neuberg, 1916] or cause swelling [Stoeltzner, 1925]. Meissner & Wöhlisch [1937] found that fibrinogen solutions had a maximum opacity when they contained 12·5 % sodium salicylate,

which agrees well with the conclusions of Pauli & Weiss [1931] on the coagulation and solution of a number of proteins. Anson & Mirsky [1933] studied the reversible denaturation of methaemoglobin and found that $0.32 M$ salicylate caused 50 % denaturation. Best [1940] found that dilute salicylate gave a birefringent precipitate with tobacco mosaic virus that was still active, whereas more concentrated solutions gave a precipitate of denatured inactive protein. In $0.5 M$ potassium salicylate a steady state of partial inactivation was set up, although inactivation was almost complete after a few hours at 30° in M solution. Tomato spotted wilt virus was much more susceptible, being completely inactivated in 1 hr. at 30° by $0.1 M$ salicylate.

Neuberg [1916] found that most of the aromatic acids had a solvent or "hydrotropic" action on proteins, but the changes that benzoic acid and its derivatives cause when they act on proteins have not been investigated. The swelling action of benzoate on pieces of dura mater was studied by Stoeltzner [1925] and Pauli & Weiss [1931], and Meissner & Wöhlisch [1937] used benzoates and hippurates as protein solvents.

Tables 8 and 9 illustrate the course of inactivation of tobacco mosaic virus and tomato bushy stunt virus by phenol. With these viruses, as with potato virus "X", in the critical range small changes in the phenol concentration have

Table 8. *The effect of phenol on tobacco mosaic virus*

Concentration of phenol M	Serological titre	Average no. of lesions per leaf at	
		10^{-4}	10^{-5}
0.416	No ppt. at 1/10,000	0	0
0.333	1/640,000	42	9
0.25	1/1,280,000	80	21
Control	1/1,280,000	75	22

0.47 % solutions of tobacco mosaic virus were exposed to the concentration of phenol stated for 20 hr. at 23°, when they were diluted for testing. In the control the phenol was added immediately before diluting. The phenol solution was adjusted to pH 7 by the addition of NaOH.

Table 9. *The effect of phenol on tomato bushy stunt virus*

Concentration of phenol M	Serological titre	Average no. of lesions per leaf at	
		10^{-4}	10^{-5}
0.416	1/80,000	0	0
0.2	1/320,000	41	7
Control	1/640,000	74	19

0.16 % solutions of tomato bushy stunt virus at pH 7 were exposed for 21 hr. at 23° to the stated concentrations of phenol, and then diluted and tested.

a large effect on the extent of the inactivation. In the absence of salt phenol does not give a precipitate with either of the viruses, but in the presence of salts a precipitate of denatured protein separates, the extent of the precipitation closely paralleling the loss of activity. As with other inactivating agents such as urea, the precipitate that separates from the inactivated tobacco mosaic virus is free from nucleic acid, whereas that from the inactivated tomato bushy stunt virus still has the nucleic acid attached.

The anisotropy of flow of tobacco mosaic virus preparations is destroyed instantly in $2 M$ sodium salicylate at 20° and in $1.4 M$ it disappears in 10–15 min. These inactive fluids remain quite clear unless they are diluted, when amorphous

precipitates separate. In more dilute salicylate the loss of anisotropy of flow is accompanied by increased opalescence, and in the range M to $0.4M$ there is precipitation of amorphous protein within a few hours. Anisotropy of flow, infectivity and serological activity, although diminished, persist for some days at 18° in $0.4M$ salicylate. As our results with salicylate are in good agreement with Best's [1940], they are not given in detail. Table 10 shows the effects of

Table 10. *The effect of sodium salicylate on tomato bushy stunt virus*

Concentration of salicylate M	Serological titre	Average no. of lesions per leaf at	
		10^{-4}	10^{-5}
1	No ppt. at 1/10,000	0	0
0·5	1/320,000	49	10
0·33	1/320,000	66	34
0·2	1/320,000	70	27
Control	1/320,000	79	37

For the first four samples 0·1 ml. of 1 % tomato bushy stunt virus was mixed with 0·1 ml. of neutralized $2M$ sodium salicylate; before the addition of the salicylate, 0·2 ml. of water was added to sample 2, 0·4 ml. to sample 3 and 0·8 ml. to sample 4. After 48 hr. at 23° the samples were diluted for testing. Salicylate was added to the control immediately before dilution and testing.

salicylate on tomato bushy stunt virus. Of the samples only that at M concentration gave any precipitate of denatured protein. In this experiment, as with others, there is evidence that $0.5M$ salicylate may be affecting infectivity without destroying serological activity. At salicylate concentrations $> M$ inactivation occurs rapidly, but the material remains in solution. Salicylate has a much greater deleterious effect on plant leaves than any of the other agents we have used. Even at dilutions as great as $0.08M$ it is necessary to wash the leaves immediately after inoculation to ensure that they shall not be damaged.

Sodium benzoate at pH 7 inactivates tobacco mosaic virus but only at higher concentrations than those necessary with salicylate; this inactivation is illustrated in Table 11. In the sample exposed to $2M$ benzoate, the anisotropy of flow disappeared in a few minutes and there was precipitation after 15 min., but in the others there was only slight loss of anisotropy of flow and increase in

Table 11. *The effect of sodium benzoate on tobacco mosaic virus*

Concentration of benzoate M	Serological titre	Average no. of lesions per leaf at	
		10^{-4}	10^{-5}
2	No ppt. at 1/10,000	0	0
1·2	1/320,000	64	16
0·8	1/640,000	90	25
Control	1/1,280,000	105	35
Control, mixed just before testing	1/1,280,000	114	37

For each of the three test samples 0·1 ml. of a 2·35 % solution of tobacco mosaic virus was added to 0·5, 0·3 and 0·2 ml. of $2.4M$ sodium benzoate solution adjusted to pH 7 with HCl. In addition samples 2 and 3 had had 0·2 and 0·3 ml. of water added. After 20 hr. at 22° they were all diluted to 2·35 ml. with water or benzoate to give a final concentration of $0.5M$ benzoate, and they were further diluted for testing 36 hr. later. In one control virus was added to benzoate at $0.5M$ and tested 36 hr. later; in the other, virus and benzoate were mixed immediately before diluting and testing.

opalescence. Sodium hippurate also inactivates tobacco mosaic virus, but still more slowly; 20 hr. exposure at 23° to $1.9M$ hippurate at pH 7 reduced the serological titre from $1/128 \times 10^4$ to $1/32 \times 10^4$, and the average number of lesions per leaf at a virus dilution of 10^{-4} from 105 to 58.

DISCUSSION

Although tomato bushy stunt virus is more susceptible to physical changes such as freezing and drying than either tobacco mosaic virus or potato virus "X", it is more resistant to the inactivating effects of the organic agents used in our experiments. Also, its denaturation often takes a different course, for whereas the commonest effect with the anisotropic viruses is the separation of nucleic acid from the protein, this is unusual with tomato bushy stunt virus. Slight changes within the particle, leading to loss of infectivity without denaturation, are also commoner with bushy stunt virus than with the others.

The number of substances that might profitably have been tested in a survey of the organic agents that inactivate plant viruses is very large, and in this work it was possible to include relatively few. These also have been tested under limited conditions, although our more detailed study of the inactivating effects of urea suggests that many factors such as pH or temperature may greatly influence inactivation, and in other conditions it is possible that their actions might be different. Apart from agents that can be regarded as oxidizing agents, acids or alkalis, Spiro, Neuberg, Hopkins, Stanley and Greenstein have, in the papers already quoted, examined the effects of over 100 substances on proteins. These cover a wide chemical range, and the most interesting substances cause irreversible changes in a few hours in neutral solution at room temperature at concentrations below $4M$. If this limit is imposed, the usual solvents, such as alcohol and acetone, whose precipitating and inactivating actions are well known even though little understood, are excluded except when they are acting on abnormally unstable proteins. Similarly urea, although so widely studied and used as a denaturant, appears to be one of the less active members of the group.

Spiro [1900; 1904], who made the first attempts to systematize the scattered observations of earlier workers, suggested that some of the agents that impede coagulation by heat do so by acting as bases, whereas others, notably the mustard oils, form soluble complexes with proteins. Several authors have adopted the second suggestion, and we have pointed out [Bawden & Pirie, 1940] that a reversible ordered association between urea and proteins is compatible with the observations that have been made on urea : protein systems. Steinhardt [1938] has suggested that if this association occurs at centres normally concerned in the formation of cross linkages between peptide chains in proteins and involves the breaking of the linkage there will, on the removal of the denaturing agent, be a greater tendency for random reformation of cross linkages the larger the number of cross linkages that has been broken. The nature of the cross linkages in proteins is still uncertain, but this picture of their reversible breakage to an extent determined by the concentration of the denaturing agent and with a corresponding change in the probability of their reformation in the original pattern seems to us a reasonably satisfactory interpretation of the observed results. On this picture the efficiency of a denaturating agent depends on its ability to form associations with and to break the normal cross linkages.

At present it is impossible to predict the action of a substance on proteins in solution from a knowledge of its physical and chemical properties, although attempts have been made to correlate the action with changes in surface tension

or dielectric constant. Thus Jirgensons [1936] stressed the significance of the dielectric constant, and showed that the concentration of a substance required to produce maximum opacity in a salt-free colloidal solution was proportional to the dielectric constant. This type of measurement, however, is complicated by the fact that it not only measures the ability of a substance to precipitate or denature a protein but also measures the ability of stronger solutions to act as protein solvents. Steinhardt [1938] concluded that there was no such proportionality, for he found that some active amides raised the dielectric constant whereas others lowered it. Hopkins [1930] drew the following conclusions from his study of denaturation of proteins by nitrogenous substances. "An amide structure is apparently necessary, but in certain relations its activity is lost. Among the ureas, mono-alkyl substitution, or unsymmetrical di-alkyl substitution, leaves the activity qualitatively intact. Symmetrical di-alkyl ureas, on the other hand, are inactive; one amino group must apparently remain unsubstituted. To judge from the case of acetyl urea, however, mono-acetyl substitution removes the activity. In biuret, allantoin and semicarbazide activity is also lost. Acid amides (acetamide and formamide) are active, but all aminoacids tried were without effect and likewise asparagine." However, as there seems to be no good reason to distinguish between the denaturing effects of nitrogenous substances and of non-nitrogenous substances such as benzoate, it is necessary greatly to extend the field in which any useful generalization must be valid. A survey of the literature and our own observations on some 15 substances suggests that the stability of a protein such as tobacco mosaic virus in a $4M$ solution of an organic substance is a more reasonable matter for comment than its instability in such an environment.

SUMMARY

The effects of alkali and of 15 simple organic substances on tobacco mosaic virus and tomato bushy stunt virus are described. Some experiments with potato virus "X" are also included. Bushy stunt virus is the most resistant to denaturation and potato virus "X" the least. The effects of alkali on tobacco mosaic virus are complex; gentle treatment may increase infectivity, slightly more severe treatment causes loss of infectivity but not loss of serological activity, and more severe treatment causes loss of all characteristic properties. With bushy stunt virus inactivation without loss of serological activity occurs over a wider pH range, and crystalline non-infective preparations can be made from alkali-treated material. Apparently similar crystalline and non-infective preparations can be isolated from expressed sap allowed to age for some months. In the presence of alkali, sodium dodecyl sulphate readily destroys all the viruses, separating the nucleic acid from the proteins. With the exception of nicotine and arginine, which form with tobacco mosaic reversible, fibrous precipitates, all the substances we have tested at concentrations below $4M$ inactivate the viruses in neutral solution. Dilute solutions of these agents are often precipitants whereas concentrated ones dissolve the products of denaturation. Inactivation of tobacco mosaic virus and potato virus "X" is usually accompanied by the separation of the nucleic acid from the protein, but inactivation of bushy stunt virus is not.

REFERENCES

Anson (1939). *J. gen. Physiol.* **23**, 239.
—— & Mirsky (1933). *J. gen. Physiol.* **17**, 399.
Bawden (1935). *Brit. J. exp. Path.* **16**, 435.
—— & Pirie (1937). *Proc. roy. Soc.* B, **123**, 274.
—— —— (1938, 1). *Brit. J. exp. Path.* **19**, 66.
—— —— (1938, 2). *Brit. J. exp. Path.* **19**, 251.
—— —— (1940). *Biochem. J.* **34**, 1258.
—— & Sheffield (1939). *Ann. appl. Biol.* **26**, 102.
Best (1936). *Aust. J. exp. Biol. med. Sci.* **14**, 323.
—— (1937). *Nature, Lond.,* **139**, 628.
—— (1940). *Nature, Lond.,* **145**, 627.
Buchner & Hoffmann (1907). *Biochem. Z.* **4**, 215.
Cohn & Conant (1926). *Proc. nat. Acad. Sci., Wash.,* **12**, 433.
Duchacek (1909). *Biochem. Z.* **18**, 211.
Edsall & Mehl (1940). *J. biol. Chem.* **133**, 409.
Eriksson-Quensel & Svedberg (1936). *J. Amer. chem. Soc.* **58**, 1863.
Fischer & Sykes (1915). *Kolloidzschr.* **16**, 129.
Fraenkel (1915). *Münch. med. Wschr.* **62**, 1401.
Fukushi (1930). *Trans. Sapporo nat. Hist. Soc.* **11**, 59.
von Furth (1896). *Arch. exp. Path. Pharmak.* **37**, 390.
Greenstein (1938). *J. biol. Chem.* **125**, 501.
—— (1939). *J. biol. Chem.* **128**, 233.
—— & Edsall (1940). *J. biol. Chem.* **133**, 397.
Grynberg (1933). *Biochem. Z.* **262**, 272.
Hebra (1892). *Mh. prakt. Derm.* **15**, 337.
Henderson (1933). *Phytopathology,* **23**, 14.
Hopkins (1930). *Nature, Lond.,* **126**, 328, 383.
Jirgensons (1936). *Koll. Beih.* **44**, 285.
Keilin & Hartree (1940). *Nature, Lond.,* **145**, 934.
Lauffer (1938). *J. phys. Chem.* **42**, 935.
—— & Price (1940). *J. biol. Chem.* **133**, 1.
Levites (1911). *Kolloidzschr.* **8**, 4.
Lundgren (1938). *J. chem. Phys.* **6**, 177.
Mauthner (1874). *Medic. Jahrb.* p. 347.
Meissner & Wöhlisch (1937). *Biochem. Z.* **293**, 133.
Mendel (1905). *Ther. Mh. (Halbmh.),* **19**, 93, 177.
Meyerhof (1918). *Biochem. Z.* **86**, 325.
Neuberg (1916). *Biochem. Z.* **76**, 107.
Pauli & Weiss (1931). *Biochem. Z.* **233**, 381.
Pedersen (1938). *C.R. Lab. Carlsberg,* **22**, 427.
Petrumkin & Petrumkin (1927). *J. gen. Physiol.* **11**, 101.
—— —— (1928). *Biochem. Z.* **201**, 185.
Plugge (1872). *Pflüg. Arch. ges. Physiol.* **5**, 538.
Runge (1834). *Ann. Phys., Lpz.* [2] **31**, 65.
Smith (1937). Textbook of plant virus diseases, p. 317. London.
—— (1940). *Science,* **91**, 199.
Spiro (1900). *Hoppe-Seyl. Z.* **30**, 182.
—— (1904). *Beitr. chem. Physiol. Path.* **4**, 300.
Sreenivasaya & Pirie (1938). *Biochem. J.* **32**, 1707.
Stanley (1935). *Phytopathology,* **25**, 899.
—— (1936). *Science,* **83**, 626.
—— & Lauffer (1939). *Science,* **89**, 345.
Steinhardt (1938). *J. biol. Chem.* **123**, 543.
Stoeltzner (1925). *Münch. med. Wschr.* **72**, 2133.
Svedberg (1937). *Nature, Lond.,* **139**, 1051.
—— & Pederson (1940). The ultracentrifuge. Oxford.
Troensgaard & Schmidt (1924). *Hoppe-Seyl. Z.* **133**, 116.
Warburg & Wiesel (1912). *Pflüg. Arch. ges. Physiol.* **144**, 465.
Wyckoff (1937). *J. biol. Chem.* **122**, 239.