The Effect of Formaldehyde and Mercuric Chloride on Tobacco Mosaic Virus

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The action of formaldehyde in killing bacteria and inactivating viruses without affecting their serological reactions is well known and widely used in the production of vaccines. The mechanism underlying this type of inactivation is by no means understood, and there is considerable contradiction in published work as to whether the changes are reversible or not. Schultz & Gebhardt (1935) reported that a *Staphylococcus* phage treated with formaldehyde could be reactivated merely by dilution and storage. Ross & Stanley (1938) reported that tobacco mosaic virus treated with formaldehyde at pH 7 could be partially reactivated by dialyzing at pH 3, that groups reacting with Folin's reagent were affected by the formaldehyde treatment and that dialysis restored these groups to their former state in degree proportional to the amount of reactivation. On the other hand, Galli & Vieuchange (1939) and Levaditi & Reinie (1939) could not regain any infectivity in neurovaccinia virus treated with formaldehyde.

Reactivation of viruses inactivated by mercuric chloride has also been claimed by various workers. Went (1937) reported that all the infectivity of tobacco mosaic virus preparations could be regained simply by dilution, and Manil (1938) claimed partial reactivation of a tobacco necrosis virus by precipitating the mercuric ions with ammonium carbonate. Similarly, Kreuger & Baldwin (1933) found that a bacteriophage of *S. aureus* which had been inactivated by mercuric chloride at pH 7 could be completely reactivated by precipitating the mercury with hydrogen sulphide.

The present paper describes experiments on the inactivating effects of formaldehyde and mercuric chloride on tobacco mosaic virus under different conditions and the failure of attempts to reactivate inactivated preparations.

EXPERIMENTAL

Purified tobacco mosaic virus (T.M.V.) in the liquid crystalline state was used in all the experiments. This was prepared by methods described by Bawden & Pirie (1943). Some preparations had been incubated with trypsin and others not; no differences were found between preparations made in the two ways.

Unless otherwise stated, virus solutions in phosphate or citrate buffer (0·06M.) were incubated at room temperature with 2% formaldehyde or 0·5% mercuric chloride. Before being tested for infectivity or serological reactions the treated virus solutions were either dialyzed thoroughly against distilled water, when the virus content was measured by micro-Kjeldahl, or diluted to below the limits at which the reagents have any effects on the plant or the virus. To avoid damage to the plants, the concentration of formaldehyde must be less than 0·5% and of mercuric chloride less than 0·05%; at pH 7 solutions of 0·25% formaldehyde and 0·01% mercuric chloride have no detectable effect on infectivity after contact for 24 hr. Infectivity tests were made by the local lesion method, with *Nicotiana glutinosa*. Folin's colour tests were made at pH 8 by the method described by Ross & Stanley (1938).

RESULTS

Effect of pH *on inactivation by formaldehyde*

Preliminary experiments showed that variation in the hydrogen-ion concentration over the pH range 3–7 had no effect on the infectivity of the virus preparations. Samples were incubated for 24 hr. at different pH values and were equally infective when inoculated after neutralization or after considerable dilution in water.

Table 1. *The effect of* pH *on inactivation of* T.M.V. *by formaldehyde and by mercuric chloride*

0·05% solutions of the virus in buffers at different pH values were incubated for 24 hr. with 2% formaldehyde or 0·5% mercuric chloride, then diluted and inoculated.

		Infectivity (numbers of lesions on six leaves)			
		Formaldehyde		Mercuric chloride	
	pH	Dilution 1/100	Dilution 1/1000	Dilution 1/100	Dilution 1/1000
	3·0	17	1	—	—
	3·5	35	6	—	—
	4·0	9	6	176	62
	5·0	3	3	122	27
	6·0	2	1	129	30
	7·0	2	0	2	0
Controls	7·0	400	55	183	45

Virus solutions in buffers of different pH values were incubated for 24 hr. with 2% formaldehyde and tested for infectivity. The results in Table 1 show that formaldehyde inactivated the virus over

the whole range tested, although the rate of inactivation was minimal around the isoelectric point, pH 3·5. With longer exposure to these conditions complete inactivation with formaldehyde can be obtained at any of these pH values. Myrbäck (1926) found that the inactivation of invertase by HCHO and $HgCl_2$ was similarly affected by pH.

Failure of attempts to reactivate tobacco mosaic virus inactivated by formaldehyde

Using the method of Ross & Stanley (1938), we attempted unsuccessfully to reactivate preparations of T.M.V. partially inactivated by formaldehyde. The results of one such experiment are given in Table 2.

Table 2. *The effect of dialysis at* pH 3·0 *on* T.M.V. *treated with* 2% *formaldehyde at* pH 7·2

Concentration of the control virus $\times 10^{-5}$ (g./ml.)	Test after incubation for 24 hr. with formaldehyde at pH 7·2. Average no. of lesions per leaf formed by: Control at varying dilutions	Test after incubation for 24 hr. with formaldehyde at pH 7·2. Average no. of lesions per leaf formed by: Treated virus at $2·0 \times 10^{-4}$ (g./ml.)	Test with formaldehyde-treated virus diluted 1/100 and stored for 3 days. Average no. of lesions per leaf formed by: Control at varying dilutions	Test with formaldehyde-treated virus diluted 1/100 and stored for 3 days. Average no. of lesions per leaf formed by: Treated virus at $2·0 \times 10^{-4}$ (g./ml.)	Test with formaldehyde-treated virus dialyzed for 3 days at pH 3·0. Average no. of lesions per leaf formed by: Control at varying dilutions	Test with formaldehyde-treated virus dialyzed for 3 days at pH 3·0. Average no. of lesions per leaf formed by: Treated virus at $2·0 \times 10^{-4}$ (g./ml.)
0·6	59	30	104	69	108	69
0·3	42	29	64	70	81	62
0·15	38	40	52	76	40	50
0·075	24	32	49	68	20	36
0·038	14	43	16	68	17	65

A 2% solution of T.M.V. was incubated for 24 hr. at pH 7·2 with 2% formaldehyde. A sample was then taken and a 1/100 dilution in water made, to reduce the concentration of formaldehyde well below the level at which it has any appreciable effect. The bulk of the sample was stored at 2°, and the remainder was used to determine the extent of inactivation. This was done by comparing on opposite half-leaves the infectivity of the sample with that of the control at various dilutions, and determining the dilution of the control which gave approximately the same number of lesions. It will be seen that infectivity of the formaldehyde-treated preparation containing $2·0 \times 10^{-4}$ g./ml. was most nearly approached by the control solution containing between $0·15 \times 10^{-5}$ and $0·3 \times 10^{-5}$ g./ml., so that the residual infectivity amounted to between $\frac{1}{70}$ and $\frac{1}{140}$ of the control.

The bulk of the formaldehyde-treated virus was dialyzed for 1 hr. against distilled water and then for 3 days at 2° against frequently changed M/10 citrate buffer of pH 3. Then it was dialyzed for 1 hr. first against distilled water and then against M/15 phosphate buffer of pH 7·2. The dialyzed preparation of formaldehyde-treated virus was then diluted to a concentration of $2·0 \times 10^{-4}$ g./ml. and compared with the control at various dilutions by rubbing over opposite half-leaves. Formaldehyde-treated virus diluted 1/100 and stored for 3 days at 2° was tested in the same way. The infectivity of both the diluted and the dialyzed sample was still most nearly approached by the control solution containing between $0·15 \times 10^{-5}$ and $0·3 \times 10^{-5}$ g./ml.

In addition, the dialyzed and the diluted samples of formaldehyde-treated virus were compared directly at 2×10^{-5} g./ml. by inoculation to the opposite halves of 80 leaves. The diluted sample gave a total of 610 and the dialyzed sample a total of 590 lesions. Thus there is no evidence that dialysis at pH 3·0 had in any way affected the infectivity of formaldehyde-treated virus. In similar experiments the loss of infectivity by incubation with 2% formaldehyde for 24 hr. at pH 7·0 varied between 98 and 99·5%. With longer incubation preparations lost more infectivity. This additional loss was prevented by adequate dilution or by dialysis, but no infectivity was regained by either treatment.

Several attempts were also made to reactivate virus which had been partially inactivated by incubation with 2% formaldehyde at pH 3·0. The drop in infectivity during incubation was less than when treatment was made at pH about 7·0, being usually between 90 and 98% after 24 hr. Again, both dilution and dialysis stopped further inactivation, but neither procedure led to any increase in infectivity.

Effect of formaldehyde treatment on Folin's pH 8·0 *colour test*

Ross & Stanley (1938) found that treating T.M.V. with formaldehyde led to a drop of about 50% in the colour value given by Folin's pH 8·0 test. They suggested that the extent of the drop in the colour value was correlated with the degree of inactivation, and they stated that dialyzing partially inactivated virus preparations at pH 3 gave an increase in the colour value corresponding to the amount of reactivation.

We have confirmed the decrease in the colour value caused by formaldehyde treatment of T.M.V.

at pH 7·0, but were unable to reverse this decrease by prolonged dialysis at pH 3·0. Neither was there any real correlation between infectivity and the colour value. If virus preparations are treated with formaldehyde for different lengths of time, there is an apparent correlation, for both colour value and infectivity decrease with increasing length of exposure. But we have found that the colour value also decreases after treatment of the virus with formaldehyde at pH 3·0. By treatment of the virus with formaldehyde at different pH values for different lengths of time, preparations can be obtained with higher colour values than others with higher infectivity. The decrease in the colour value after a prolonged formaldehyde treatment of the virus at pH 3 0 is about 20%, whereas at pH 7 it is about 50%. Both the decrease in colour value and of infectivity proceed more rapidly when formaldehyde treatment is carried out at pH 7·0 than at pH 3·0, so that if the virus is incubated for equal lengths of time with formaldehyde at the two pH values there is again an apparent correlation between the colour value and infectivity. But if the virus is incubated with formaldehyde for longer periods at pH 3·0 than at pH 7·0, the infectivity may be lower though the colour value may be higher. For example, in one test when 1% solutions of the virus were incubated with 2% formaldehyde at the two pH values for 24 hr., at pH 7·0 the colour value was 55% and the number of lesions 4% of the original, and at pH 3·0 the colour value was 90% and the number of lesions 35%. However, the virus preparation treated with formaldehyde at pH 7·0 for 16 hr. had a colour value of 65% and the number of lesions 7% of the original, whereas the preparation treated for 72 hr. at pH 3·0 had a colour value of 80% although the number of lesions was only 1·5% of the original.

Ross & Stanley (1938) attributed the decrease in Folin's pH 8·0 colour value after formaldehyde treatment of T.M.V. at pH 7·0 to changes in tryptophan. They deduced this from the fact that the colour value given by tryptophan is decreased after incubation at pH 7·0 with formaldehyde, whereas the colour value of tyrosine, the only other constituent of the virus known to give the reaction, remains unaltered.

We have tested the effect of formaldehyde on tryptophan and tyrosine at pH 8·0 and 3·0. 0·2% solutions of the amino-acids in buffers at pH 3·0 and 8·0 were incubated for 48 hr. at 37° with 2% formaldehyde. The crystalline precipitate that appeared in the tryptophan treated with formaldehyde at pH 8·0 was dissolved by heating before the testing for Folin's colour value. The colour value of tryptophan was reduced to about 60% after treatment at both pH values, whereas that of tyrosine was unaffected. The fact that both tryptophan and T.M.V. have their colour value reduced when treated with formaldehyde in acid and alkaline conditions supports the view that changes in tryptophan occur during formaldehyde treatment of the virus. Nevertheless, the interpretation is by no means straightforward. The colour value of isolated tryptophan falls by approximately the same amount after treatment with formaldehyde at pH 3·0 or 8·0, whereas that of the virus falls much more at pH 7·0 than at pH 3·0. If the tyrosine:tryptophan ratio in T.M.V. is about 0·8 (Knight & Stanley, 1941) and the ratio of Folin's pH 8·0 colour value given by tyrosine to that given by an equal weight of tryptophan is about 1·3, the colour value of T.M.V. treated with formaldehyde at pH 3·0 falls to a value that would be expected from the behaviour of isolated tryptophan; however, the fall after treatment with formaldehyde at pH about 7·0 is much greater than expected. There seem to be two equally possible explanations: the virus may contain certain other constituents which react with Folin's reagent and are affected by treatment with formaldehyde at pH 7·0 but not at pH 3·0; or tryptophan as combined in the virus is affected more by formaldehyde treatment at pH 7·0 than when it is free. There is evidence that the manner in which constituents are combined in a protein does affect the colour value, for heat denaturation increases by about 40% the colour value given by T.M.V., and a similar effect of denaturation has been described with pepsinogen (Herriott, 1938). Therefore, the colour value given by a protein does not seem to be simply a summation of the values of individual constituents, but also depends on the manner in which these are arranged. Because of this it is impossible to interpret the changes in the virus on the basis of changes measured on isolated amino-acids.

Wadsworth & Pangborn (1936) found that the crystalline product formed during formaldehyde treatment of tryptophan at pH 8·0 was 3, 4, 5, 6-tetrahydro-4-carboline-5-carboxylic acid, which was obtained by Jacobs & Craig (1936) by treating tryptophan with formaldehyde in acid medium. We have obtained a crystalline product from T.M.V. treated with formaldehyde at pH 7·5 similar to that formed during incubation of tryptophan with formaldehyde at pH 8·0. The formaldehyde-treated virus was dialyzed, denatured by heat, incubated with 0·5% commercial trypsin for 3 days at 37° and then left for 6 days at 2°. No attempt was made to identify the crystals, but they were not formed in control virus solutions treated similarly but without formaldehyde.

Effect of formaldehyde treatment of T.M.V. on the Van Slyke test for amino-nitrogen

10 mg. of T.M.V. was found by the Van Slyke method for estimation of free amino-nitrogen (Van Slyke, 1929) (shaking time 4 min.) to give 8·5 mm.

(at 20°) increase of pressure compared with a blank. This corresponds to a free amino-nitrogen content equivalent to 0·8 % of the total nitrogen. Treatment with formaldehyde at pH 7·4 for 24 hr. decreased the value given by the Van Slyke test to about 60 % of the value for untreated virus, and this remained unaffected by 3 days' dialysis at pH 3·0. This agrees with the results of Ross & Stanley (1938). Non-infective virus produced by formaldehyde treatment at pH 3·0 for 3 days gave the same value in the Van Slyke test as control virus, which suggests that changes in groups responsible for the test are not necessarily associated with loss of infectivity caused by formaldehyde. It must be emphasized, however, that in view of the small number of groups giving the test, small differences would not be detectable, and that the test shows only changes not reversible by the treatment which it involves.

Inactivation of T.M.V. by mercuric chloride

Table 1 shows that mercuric chloride inactivates the virus only over a narrow range of pH values, having little effect in acid conditions. Loss of infectivity as a result of formaldehyde treatment is not accompanied by any loss of serological activity, and non-infective preparations of the virus give the same titres in the precipitin test as fully infective controls. Treatment with mercuric chloride at pH's above 6·0 leads to a reduction of infectivity and serological activity; a change also occurs in physical properties, for treated solutions become increasingly viscous and lose their birefringence.

Table 3. *The effect of mercuric chloride at* pH 7·0 *on the infectivity and serological activity of T.M.V.*

Time of incubation (hr.)	Infectivity; lesions as percentage of the number formed by the control (538)	Serological test; precipitation end-point dilution
Control	100	1/6400
0	137	1/6400
3	127	1/3200
8	59	1/1600
24	14	1/1600
48	11	1/1600
72	4	1/1600

Table 3 shows the results of one experiment in which 0·4 % solutions of T.M.V. were incubated with 0·5 % mercuric chloride at pH 7·0 and then diluted 1/100 and inoculated into plants. Six leaves were inoculated with each preparation. It will be seen that in the early stages of incubation there was a significant drop in serological titre, while infectivity was actually greater than that of the control. After prolonged incubation the ratios between infectivity and serological activity were reversed, for infectivity fell steadily without any corresponding reduction of precipitin titre. From such preparations material could be removed by centrifugation at 10,000 r.p.m., leaving serologically active material in the supernatant fluid, which still showed anisotropy of flow.

A somewhat similar result was obtained by Bawden & Pirie (1940) with T.M.V. treated with alkali, when gentle treatment often led to an increase in infectivity, while more vigorous treatment led to a decrease. They regarded the increase as a result of the disaggregation of large virus aggregates into smaller infective units.

It was shown in Table 1 that inactivation by mercuric chloride occurs only at pH value higher than 6·0, and then, as shown in Table 3, it proceeds gradually. It can be stopped at any stage by acidification, by dilution sufficient to reduce the concentration of mercuric chloride below 0·01 %, or by adding sufficient quantities (2–6 %) of certain salts. Some of the salts, e.g. $(NH_4)_2CO_3$, $(NH_4)_2SO_4$ or $(NH_4)_2S$, act by precipitating Hg^{++}, and others, e.g. $BaCl_2$ or $Ca(NO_3)_2$, possibly by acidification of the solutions. There are, however, some salts, such as NaCl or KCl, which neither precipitate Hg^{++}, nor acidify the solutions, but which completely prevent inactivation. We have been unable to find any indication that these treatments reversed inactivation. By dilution, acidification or by addition of certain salts, inactivation could be stopped at any stage, but virus already inactivated could not be reactivated. Samples of the virus incubated with 0·5 % mercuric chloride at pH 7·0 showed the same infectivity when tested immediately after any of these treatments as when tested 24 hr. later. In some cases, especially during the stage when inactivation proceeded most rapidly, acidification or addition of some salts resulted in higher infectivity than that given by dilution. This difference could be interpreted by the assumption that such treatments stop the progress of inactivation more suddenly than dilution.

DISCUSSION

The main points that call for discussion are the discrepancies between our results and those of other workers, who claimed that T.M.V. inactivated with formaldehyde or with mercuric chloride could be reactivated. One possible explanation is that we have worked with a strain of virus which behaves differently, but this is unlikely, as few differences have been found in the behaviour of different strains of T.M.V. The discrepancies probably arise from the unreliability of infectivity tests as measurements of the amount of active virus present in different inocula, because the number of lesions produced by an inoculum can be affected by many other factors.

Many substances are known, such as trypsin, serum proteins and some plant constituents, which act as inhibitors of infectivity. When present in inocula these greatly reduce infectivity, and by adding and removing them from virus preparations apparently complete inactivation and reactivation can be produced (Bawden, 1943). Whether such inhibitions are caused by affecting the host or by forming non-infective complexes with the virus is still unsettled, but there is no evidence that the inhibitors produce any changes within the virus particles. In concentrations over 0·01%, mercuric chloride can act as such an inhibitor at pH below 6·0, and this probably explains Went's (1937) claim of complete reactivation by dilution in water of solutions of T.M.V. containing mercuric chloride. Went did not control the pH of her virus-mercuric chloride mixtures, which were probably acid, a condition in which true inactivation does not proceed, although inhibition might be complete and there would probably be obvious damage to the inoculated leaves. When dilution was such that the concentration of mercuric chloride was reduced below the inhibiting level, the original infectivity would be regained, and it would look as though the virus had been reactivated.

Similarly, the presence of small amounts of inhibitors in the preparations used by Ross & Stanley (1938) could explain the apparent small amount of reactivation gained by dialyzing formaldehyde-treated virus preparations. It is possible that dialysis at pH 3·0 either removed or destroyed some impurity which was reducing infectivity. This could also explain the change in the Folin colour value after dialysis, which we were unable to confirm in our tests.

Even in the absence of inhibitors, however, infectivity does not depend solely on the weight of active virus, but also on the number of virus particles. It is known that T.M.V. particles can aggregate linearly to form large rods (Bawden & Pirie, 1937), and this affects the infectivity. That this effect is partially reversible is suggested by the increase of infectivity frequently given by short exposures to alkali (Bawden & Pirie, 1940) or mercuric chloride (cf. Table 3). The increase caused by mercuric chloride was often greater than that shown in Table 3, for in this test the concentration was such that inactivation was also proceeding rapidly. The presence of more dilute mercuric chloride has often increased the infectivity to more than twice that of control virus solutions. Thus the discrepancies between our results and those of Ross & Stanley (1938) could also have resulted from the use of virus preparations in different degrees of aggregation, with their degrees of aggregation being differently affected by the processes which led to an apparent reactivation of infectivity.

SUMMARY

1. Tobacco mosaic virus was inactivated by 2% formaldehyde at all pH values between 3 and 7·5; the rate of inactivation was minimal at pH 3·5. Inactivation could be stopped at any stage by dilution or dialysis, but there was no evidence that inactivated virus regained infectivity by these treatments.

2. Loss of infectivity caused by formaldehyde does not depend on changes in groups giving the Van Slyke test for amino-nitrogen, for preparations inactivated by formaldehyde treatment at pH 3·0 give the same value as control virus. Formaldehyde treatment at pH near 7·0 leads to a greater fall in the Folin pH 8·0 colour value than treatment at pH 3·0. There is no real correlation between the decrease of infectivity and of the colour value.

3. Mercuric chloride in sufficient concentrations acts as an inhibitor of infectivity. At pH values greater than 6·0 it causes loss of infectivity and serological activity. Dilution, acidification or addition of certain salts prevent inactivation or interrupt its progress at any stage, but there is no evidence that any of these treatments can reverse it.

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