THE PARTIAL REACTIVATION OF FORMOLIZED TOBACCO MOSAIC VIRUS PROTEIN

By A. FRANK ROSS and W. M. STANLEY

(From the Department of Animal and Plant Pathology of The Rockefeller Institute for Medical Research, Princeton, New Jersey)

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Considerable evidence has accumulated indicating that virus activity is a specific property of the tobacco mosaic virus protein (1), yet little information is available on the relationship between protein structure and virus activity. Certain reagents inactivate the virus, and at least part of the changes they bring about in the protein molecule have been determined (2). In no case, however, has the nature of the specific change that causes loss of activity been established. The reactivation of inactivated virus would appear to be one of the simplest means of obtaining direct evidence relating virus activity to protein structure. Should it prove possible to alter the protein by some chemical means that also destroys virus activity, to demonstrate that the protein is altered and to establish the chemical nature of the change, and then to restore the protein molecule to its original form with an accompanying restoration of virus activity, it would serve to correlate directly virus activity with the structure of the protein.

It has been shown that tobacco mosaic virus protein inactivated by hydrogen peroxide, ultraviolet light, formaldehyde, or nitrous acid is only slightly altered and retains certain chemical and serological properties characteristic of the active virus protein (2). The inactive proteins can be crystallized, are soluble, are still native, and give positive precipitin reactions with the sera of animals injected with active virus preparations. Dr. Lauffer[1] has found that the stream double refraction of formolized virus protein is the same or slightly less than that of active virus protein, and it has been shown that the sedimentation constant of tobacco mosaic virus protein inactivated

[1] Personal communication (see also *J. Biol. Chem.*, 1938, **123**, 507).

by hydrogen peroxide, formaldehyde, or nitrous acid (3) is practically the same as that of the active protein. These reagents must then cause only minor changes in the protein molecule, hence it seemed possible that the reactions caused by some of them might be reversible. The reaction of formaldehyde with proteins or with amino acids has been shown to be partially reversible by Holden and Freeman (4) and by Wadsworth and Pangborn (5). Anderson (6) has shown that, while the condensation between formaldehyde and gelatin is fairly stable in alkaline solutions, it is less stable in acid solutions. Schultz and Gebhardt (7) stated in a preliminary note that they had obtained reactivation of formolized bacteriophage merely by dilution. The reaction between the virus protein and formaldehyde was, therefore, selected as the one most likely to be reversible as well as one of a type that might permit the determination of the chemical changes that occur. The present paper records the results obtained in a study of the inactivation of tobacco mosaic virus protein with formaldehyde and the reactivation of the formolized protein.

EXPERIMENTAL

Preparation of Virus Protein.—The tobacco mosaic virus protein used in the present experiments was isolated from diseased Turkish tobacco plants either by a chemical method (8) or by means of an ultracentrifuge (9). In the first method the virus was precipitated from the filtered dilute dipotassium phosphate extract of the frozen macerated plants with ammonium sulfate and the precipitate was dissolved in M/10 phosphate buffer at pH 7. The precipitation was repeated until the filtrate remained colorless, after which the virus protein was adsorbed on celite at pH 4.5 and then eluted at pH 7. By working in a cold room and keeping all preparations below 5°C., colorless preparations were obtained without the use of calcium oxide. In the second method the protein was isolated from the filtered extract by alternate high and low speed centrifugation. Preparations were dialyzed at least 24 hours against flowing distilled water before they were used and, as they contained no detectable amounts of non-protein nitrogen, protein estimations were calculated from total nitrogen determinations.

Determination of Activity.—The half-leaf method of inoculation (10, 11) as used in this laboratory (12) was employed, with the exception that whole leaves were inoculated when testing for complete or nearly complete inactivation. *Nicotiana glutinosa* L. was selected for use as the test plant in most of the experiments because it was found to be affected less by the toxic action of high concentrations of inactive virus protein than was *Phaseolus vulgaris* L., var. Early Golden Cluster. Since it proved necessary to determine to what extent the production of toxic

protein accounts for the loss of activity, the effect of known amounts of inactive protein on the number of lesions obtained with virus protein was studied. Mixtures were made of active virus protein with various amounts of formaldehyde-inactivated virus protein, hydrogen peroxide-inactivated virus protein, or egg albumin, and the virus activity of the mixtures compared with that of active virus protein on both the above mentioned test plants by means of the half-leaf method. It may be seen from the results presented in Table I that a concentration of 10^{-3} gm. of inactive protein per cubic centimeter had little if any effect on activity measurements when *Nicotiana glutinosa* was used, but that the effect was appreciable

TABLE I

Effect of Different Concentrations of Various Inactive Proteins on the Activity of Tobacco Mosaic Virus Protein

Inactive protein used	Concentration (gm. protein/cc.) of mixtures		*Nicotiana glutinosa*				*Phaseolus vulgaris*			
			No. of half-leaves	Lesions		A/B × 100†	No. of half-leaves	Lesions		C/D × 100†
	Active	Inactive		A Mixture	B Control*			C Mixture	D Control*	
Formolized virus protein	10^{-5}	10^{-2}	27	371	737	50.5	33	337	1049	31.8
	10^{-5}	10^{-3}	31	917	1090	83.8	27	637	876	72.7
" " "	10^{-5}	10^{-2}	33	579	929	62.3				
	10^{-5}	10^{-3}	30	529	501	105.6	35	529	765	69.1
	10^{-5}	10^{-4}	34	900	892	100.9	24	342	402	82.5
H_2O_2-inactivated virus protein	10^{-5}	10^{-2}	29	207	543	38.1	32	79	566	13.9
	10^{-5}	10^{-3}					22	211	577	36.5
" "	10^{-5}	10^{-2}	24	153	597	25.6	38	460	3345	13.8
Egg albumin	10^{-5}	10^{-2}	27	807	1194	66.7	32	248	1018	24.5
	10^{-5}	10^{-2}	27	894	1352	66.3	32	558	1805	30.8
" "	10^{-5}	10^{-2}	31	547	893	61.3	41	2655	7392	35.9

* All controls contained 10^{-5} gm. active protein per cubic centimeter.

† The figures in this column are regarded as a measure of the relative activities of the mixtures when tested on the host indicated.

with *Phaseolus vulgaris*. The formolized virus protein caused a smaller reduction in activity than did hydrogen peroxide-inactivated virus protein and had about the same or a slightly greater effect than egg albumin. Regardless of whether the action of the inactive protein was an effect on the plant (13, 14), an effect on the virus, or a mechanical effect, it is evident that this production of a mildly toxic protein cannot account for the failure of formolized tobacco mosaic virus protein to exhibit virus activity and also that the effect of inactive protein on virus activity can be measured and a correction factor determined.

In the experiments to be reported, the activity of a given preparation was

determined by inoculating against a control containing 10^{-5} or 10^{-6} gm. of active virus protein per cubic centimeter and the results interpreted on the assumption that over the range used the number of lesions is proportional to the virus concentration. That such a proportionality exists over the range of 10^{-5} to 10^{-6} gm./cc. has been shown by several investigators (15, 16). It was necessary to introduce a correction factor when preparations containing large amounts of inactive protein were used. This factor was obtained from data such as those presented in Table I and in Fig. 1. Curve A of Fig. 1, a dilution curve of one of the preparations used,

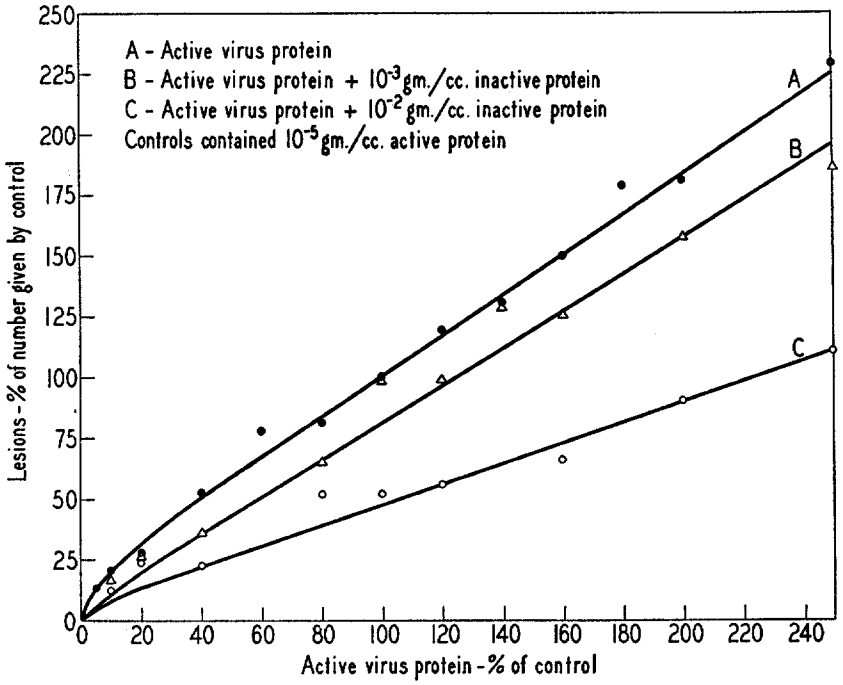

FIG. 1. The effect of formaldehyde-inactivated virus protein on the activity of tobacco mosaic virus protein.

was obtained by comparing the number of lesions given by different amounts of active virus protein with the number given by a control containing 10^{-5} gm. of active protein per cubic centimeter when inoculated to *Nicotiana glutinosa* using the half-leaf method. The number of lesions obtained in each case is expressed as per cent of those caused by the control. The figure shows that such a curve is not far from a straight line of slope 1 and that the error introduced in assuming that such is the case is not great. The other two curves were obtained in the same manner, except that definite amounts of completely inactive formolized virus

protein were added to each inoculum (but not to the controls). As may be seen from Fig. 1, a concentration of 10^{-3} gm. of inactive protein per cubic centimeter had but slight effect on the dilution curve of active virus. In many preparations this amount had no measurable effect on the virus activity. A concentration of 10^{-2} gm. of inactive protein per cubic centimeter caused a constant percentage reduction of about 50 per cent in the number of lesions that was obtained. Other preparations gave similar curves. Therefore, with preparations containing 10^{-3} gm. of inactive protein per cubic centimeter no correction was made, while with those containing 10^{-2} gm./cc. the figures for apparent activity were multiplied by a factor of 2. In most experiments involving the comparison of different samples, an attempt was made to dilute the samples so that approximately the same portions of the curves were used.

Inactivation.—Unless otherwise stated, inactivation was allowed to proceed at room temperature in mixtures of approximately 2 per cent protein and 2 per cent formaldehyde in M/10 phosphate buffer at pH 7. Immediately after mixing the reactants, a sample of the mixture was removed, diluted to 10^{-5} or 10^{-6} gm. of protein per cubic centimeter, and inoculated against a control of the same concentration. At intervals samples were removed for immediate dilution and inoculation and for dialysis against flowing cold distilled water for 6 hours in a Kunitz-Simms rocking type apparatus (17) to stop the reaction by removal of free formaldehyde. After dialysis the samples were analyzed for nitrogen and diluted to suitable concentrations for inoculation against controls consisting of 10^{-5} or 10^{-6} gm. of the original active virus protein per cubic centimeter. Data on rate of inactivation are given in Tables II and III. The latter table presents the data obtained in an experiment in which duplicate samples were used and is included as an illustration of the uniformity of results that may be expected under the conditions of the experiment. It may be seen that, in view of the accuracy of activity determinations (12) and of other factors, the results agree quite well. During the first 12 to 18 hours in which over 99 per cent of the protein is inactivated, the rate of inactivation follows roughly that of a monomolecular reaction. However, a much greater length of time is required for complete inactivation than would be predicted on such a basis. For this and other reasons it is not implied that the reaction is monomolecular, and the expression is used merely to describe the general shape of the inactivation curve. There are not sufficient data at hand to permit a determination of the exact type of the reaction.

Although it was found that dialysis removed the excess formaldehyde quite rapidly from the reaction mixture, it appeared desirable to determine whether or not the dialyzed preparations that were used for inoculation contained sufficient quantities of free formaldehyde to affect their activities. Tests on the dialysates showed that after 5½ hours of dialysis against cold distilled water, the solutions con-

TABLE II

Rate of Inactivation of Tobacco Mosaic Virus Protein (1.86 Per Cent) by 2 Per Cent Formaldehyde at pH 7.0 and at Room Temperature

Experiment	Time	No. of half-leaves	Concentration at which inoculated (gm. protein/cc.)		Lesions			Activity remaining
			Sample	Control	A Sample	B Control	A/B × 100*	
	hrs.							*per cent*
	0†	27	10^{-6}	10^{-6}	326	328	100	100
	6†	28	10^{-5}	10^{-6}	599	497	120.7	12
	12†	14	10^{-4}	10^{-6}	419	385	108.8	1
	24†	32	10^{-3}	10^{-6}	108	248	43.6	0.04
4‡	48†	40	10^{-2}		22			
	48	38	10^{-2}		60			
	72	42	10^{-2}		12			
	96	40	10^{-2}		3			
	192	38	10^{-2}		0			0.0
	0†	13	10^{-5}	10^{-5}	183	211	87	87
	6†	14	10^{-4}	10^{-5}	127	218	58	6
	6	25	2×10^{-4}	10^{-5}	406	477	85	4
5	12†	28	10^{-4}	10^{-6}	47	58	81	0.8
	12	27	2×10^{-4}	10^{-5}	628	541	116	0.6
	24†	30	10^{-3}	10^{-6}	34	112	30	0.03
	24	28	5×10^{-3}	10^{-5}	110	267	41	0.01
	192	26	10^{-2}		0			0.0
	5	30	10^{-4}	10^{-5}	1704	741	230.0	23
	10	28	10^{-3}	10^{-5}	192	548	35.0	0.35
	24	30	10^{-2}	10^{-5}	153	948	16.1	0.03
12‡	96	30	10^{-2}		6			
	120	34	10^{-2}		2			
	144	30	10^{-2}		0			0.0
	168	26	10^{-2}		0			0.0
	5	33	10^{-5}	5×10^{-6}	153	499	31.3	15
	8	32	10^{-4}	5×10^{-6}	340	479	71.0	3.5
20‡	12	34	10^{-3}	5×10^{-6}	221	489	45.4	0.23
	24	34	10^{-2}	5×10^{-6}	30	463	6.5	0.006
	48	34	10^{-2}	10^{-6}	4	230	1.7	0.0003

* The figures in this column represent the relative activities of the samples when compared with the controls at the concentrations indicated with *Nicotiana glutinosa* as test plant.

† HCHO not removed from samples used for inoculation.

‡ Ultracentrifugally prepared virus protein used in these experiments.

tained less than 10^{-5} gm. of free formaldehyde per cubic centimeter. That this amount was too small to affect the activity of the preparations is evident from the data presented in Tables II and III, for many of the samples were used for inoculation without removal of the excess formaldehyde. Samples removed immediately after mix-

TABLE III

Rate of Inactivation of Tobacco Mosaic Virus Protein (1.86 Per Cent) by 2 Per Cent Formaldehyde at pH 7.0 and at Room Temperature

Hours	Sample*	Concentration (gm. protein/cc.)		No. of half-leaves	Lesions			Activity remaining
		Sample	Control		C Sample	D Control	C/D × 100†	
								per cent
0	A‡	10^{-5}	10^{-5}	30	1063	919	116.8	117
	B‡	10^{-5}	10^{-5}	28	1106	1108	99.8	100
6	A‡	10^{-4}	10^{-5}	29	684	1328	51.5	5
	B‡	10^{-4}	10^{-5}	28	821	1136	72.2	7
	A	10^{-4}	10^{-5}	29	776	1170	66.3	7
	B	10^{-4}	10^{-5}	29	760	983	77.3	8
12	A‡	10^{-3}	10^{-5}	28	643	1080	59.3	0.6
	B‡	10^{-3}	10^{-5}	29	529	1124	47.1	0.5
	A	10^{-3}	10^{-5}	26	474	1162	40.8	0.4
	B	10^{-3}	10^{-5}	29	741	1251	59.2	0.6
24	A	10^{-2}	10^{-5}	27	360	669	53.8	0.11
	B	10^{-2}	10^{-5}	26	284	698	40.7	0.08

* Samples A and B were prepared from the same virus preparation and were adjusted to the same protein, formaldehyde, phosphate, and hydrogen ion concentrations.

† The figures in this column represent the relative activities of the samples when compared with the controls at the concentrations indicated with *Nicotiana glutinosa* as test plant.

‡ Excess formaldehyde not removed from samples used for inoculation.

ing and containing 10^{-5} gm. free formaldehyde per cubic centimeter when inoculated were fully as infectious as the corresponding controls containing no formaldehyde. Further inspection of the tables reveals that solutions containing higher concentrations of free formaldehyde were practically as active as corresponding samples from which form-

aldehyde had been removed by dialysis. A formaldehyde concentration of 10^{-4} gm./cc. had little or no effect and 10^{-3} gm./cc. reduced virus activity only slightly.

The data presented in this section, together with those on stream double refraction and sedimentation constants of the formolized virus protein, indicate that the inactivation of the virus by formaldehyde is not due to a toxic action of the formaldehyde on the test plant, to the production of a toxic protein, or to aggregation, and make it seem quite probable that inactivation is due to an alteration of the structure necessary for virus activity.

Reactivation.—Preliminary experiments indicated that a limited amount of reactivation could be obtained by incubation of the inactivated protein with dimethyldihydroresorcinol (5) or with histidine (4) or by dialysis. As dialysis proved most effective, the other methods were abandoned. Although it was apparent that some reactivation was secured by dialysis against distilled water at about pH 6.5, there was an indication that the reactivation was more rapid when the dialysis was carried out at pH 3, hence the latter treatment was used as the routine reactivation process. The results of several experiments indicated that with most preparations maximum activity was obtained in about 3 days. While some of the more inactive preparations did not quite reach a maximum in that time, a prolonged dialysis period caused an appreciable decrease in activity, hence a 3 day period of dialysis was adopted for routine use.

Partially inactivated preparations of tobacco mosaic virus protein were obtained by removing portions at intervals, as described in the preceding section. Portions of these were subjected to the reactivation process, or samples to be reactivated were removed from the reaction mixture simultaneously with the other samples and subjected to the reactivation process immediately. As the results were the same in both cases, the method of sampling was not recorded in the tables. For reactivation the preparations of formolized virus protein were adjusted to pH 3 by dialysis against approximately 0.001 M phosphate-citrate-HCl buffer at pH 3 in a Kunitz-Simms apparatus and the dialysis at that pH was continued for 3 days. The samples were then dialyzed against phosphate buffer to bring them to pH 7, and then against distilled water to remove the phosphate. They were then analyzed for nitrogen and portions adjusted to M/10 phosphate and to protein concentrations suitable for inoculating against controls containing 10^{-5} or 10^{-6} gm. of the original active virus per cubic centimeter. When active

virus preparations were subjected to the above treatment, their activity was either unchanged or decreased less than 20 per cent.

It became apparent early in the investigation that the amount of reactivation obtainable was dependent to some degree upon the extent to which inactivation had proceeded. In Table IV are included the data on reactivation of preparations retaining approximately 10 per cent of their original activity. In no case was the activity restored in full, but some reactivation was obtained in all cases, although in certain instances the differences may not be significant. The values given for the activity of the preparations are numbers calculated from the data and are regarded as a measure of the order of magnitude of the true activities. In most cases the differences are too large and obtained too often to be accounted for by errors of the method. It should be noted that Tables IV–VII record representative data on attempts at reactivation and do not give only the most favorable data. The numbers in the last column of each of Tables IV–VI are the ratios of the activities of the reactivated samples to those of the corresponding samples before reactivation. Each represents the number of times the activity of the designated sample was increased by the reactivation process.

Similar data on preparations possessing about 1 per cent of their original activity are presented in Table V. It may be seen that the results are more consistent and the differences obtained considerably larger. A 10-fold increase in activity was obtained in most cases, although the actual amount of reactivation in terms of active protein "created" is less than was obtained with some of the more active preparations. Due to the fact that it is possible to inoculate at different levels of protein concentration, it is as easy to distinguish between 1 per cent and 2 per cent active preparations as it is to distinguish between 50 per cent and 100 per cent active preparations. An increase from about 1 per cent activity to 10 per cent activity is quite significant and indicates a marked increase in the actual activity of the virus preparation.

As may be seen from the results that are presented in Table VI, essentially the same degree of reactivation was obtained with virus protein preparations possessing about 0.1 per cent of the original activity. In these experiments it was necessary to introduce a cor-

TABLE IV

Reactivation of Formolized Tobacco Mosaic Virus Protein Preparations Possessing Approximately 10 Per Cent of the Original Activity

Experiment	Before reactivation							After reactivation							Reactivation (No. of times increase in activity)
	No. of half-leaves	Concentration (gm. protein/cc.)		Lesions			Activity (per cent of original)	No. of half-leaves	Concentration (gm. protein/cc.)		Lesions			Activity (per cent of original)	
		Sample	Control	A Sample	B Control	A/B × 100*			Sample	Control	C Sample	D Control	C/D × 100*		
4†	28	10^{-5}	10^{-6}	599	497	120.7	12	31	10^{-5}	10^{-6}	765	533	143.6	14	1.2
5	25	2×10^{-4}	10^{-5}	406	477	85.1	4	30	2×10^{-5}	10^{-5}	347	558	62.2	31	7.8
6‡	29	10^{-4}	10^{-5}	776	1170	66.3	7	31	5×10^{-5}	10^{-5}	917	979	93.8	19	2.7
6‡	29	10^{-4}	10^{-5}	760	983	77.3	8	30	5×10^{-5}	10^{-5}	847	933	52.2	10	1.2
8§	28	10^{-4}	10^{-5}	1310	1106	117.5	12	29	10^{-5}	10^{-5}	602	733	82.1	82	6.8
8‖	26	10^{-4}	10^{-5}	1356	998	135.9	14	34	10^{-5}	10^{-5}	134	588	22.8	23	1.6
9	29¶	10^{-4}	10^{-5}	928	1901	48.8	5	28**	10^{-5}	10^{-5}	334	1292	25.9	26	5.2
9§	25¶	10^{-4}	10^{-5}	623	1036	60.1	6	36**	10^{-5}	10^{-5}	390	2622	14.9	15	2.5
9‖	28¶	10^{-4}	10^{-5}	911	986	92.4	9	16**	10^{-5}	10^{-5}	257	573	44.9	45	5.0
10**	30	10^{-4}	10^{-5}	1065	847	126.8	13	29	10^{-5}	10^{-5}	796	1527	52.1	52	4.3
18†	31	10^{-4}	2×10^{-6}	858	448	191.5	4	30	10^{-5}	2×10^{-6}	451	1454	31.0	6	1.6
20†	33	10^{-5}	5×10^{-6}	153	499	31.3	16	30	10^{-5}	5×10^{-6}	522	1102	47.3	24	1.4

* The figures in this column represent the relative activities of the samples when compared with the controls at the concentration indicated.

† Ultracentrifugally prepared virus protein used in these experiments.

‡ Duplicates.

§ Inactivated by 5 per cent HCHO at pH 6.0.

‖ " " 10 " " " " " 7.0.

¶ *Phaseolus vulgaris* used for test plant. *Nicotiana glutinosa* used in all other cases.

** Inactivated by 5 per cent HCHO at pH 7.0.

TABLE V

Reactivation of Formolized Tobacco Mosaic Virus Protein Preparations Possessing Approximately 1 Per Cent of the Original Activity

Experiment	Before reactivation							After reactivation							Reactivation (No. of times increase in activity)
	No. of half-leaves	Concentration (gm. protein/cc.)		Lesions			Activity (per cent of original)	No. of half-leaves	Concentration (gm. protein/cc.)		Lesions			Activity (per cent of original)	
		Sample	Control	A Sample	B Control	A/B × 100*			Sample	Control	C Sample	D Control	C/D × 100*		
4†	14	10^{-4}	10^{-6}	419	385	108.8	1.1	27	10^{-5}	10^{-6}	448	382	117.2	12	12
6‡	26	10^{-3}	10^{-5}	474	1162	40.8	0.4	27	2×10^{-4}	10^{-5}	640	772	82.9	4	10
6‡	29	10^{-3}	10^{-5}	741	1251	29.2	0.3	30	2×10^{-4}	10^{-5}	421	648	65.0	3	10
8§	27	10^{-3}	10^{-5}	1672	1206	138.6	1.4	35	10^{-4}	10^{-6}	1902	1431	133.5	13	9
8§	30	10^{-3}	10^{-5}	500	495	101.0	1.0	25	10^{-3}	10^{-5}	1395	833	167.0	2	2
8‖	30	10^{-3}	10^{-5}	2175	1691	128.6	1.3	29	10^{-4}	10^{-5}	1340	1003	133.6	13	10
8‖	33	10^{-3}	10^{-5}	2277	1497	152.1	1.5	30	10^{-4}	10^{-5}	1109	1089	101.9	10	7
10¶	27	10^{-3}	10^{-5}	1383	976	140.7	1.4	29	10^{-4}	10^{-5}	689	656	105.3	10	7
20†	32	10^{-4}	5×10^{-6}	340	479	71.0	3.5	24	10^{-5}	5×10^{-6}	184	655	28.3	14	4

* The figures in this column represent the relative activities of the samples when compared with the controls at the concentrations indicated with *Nicotiana glutinosa* as test plant.

† Ultracentrifugally prepared virus protein used in these experiments.

‡ Duplicates.

§ Inactivated by 5 per cent HCHO at pH 6.0.

‖ " " 10 " " " " " 7.0.

¶ " " 5 " " " " " 7.0.

TABLE VI

Reactivation of Formolized Tobacco Mosaic Virus Protein Preparations Possessing Approximately 0.1 Per Cent of the Original Activity

Experiment	Before reactivation							After reactivation							Reactivation (No. of times increase in activity)
	No. of half-leaves	Concentration (gm. protein/cc.)		Lesions			Activity (per cent of original)	No. of half-leaves	Concentration (gm. protein/cc.)		Lesions			Activity (per cent of original)	
		Sample	Control	A Sample	B Control	A/B × 100*			Sample	Control	C Sample	D Control	C/D × 100*		
4†	30	10^{-3}	10^{-6}	108	248	43.6	0.04	14	10^{-4}	10^{-6}	150	169	88.8	0.9	22
6‡	27	10^{-2}	10^{-5}	360	669	53.8	0.10	30	10^{-3}	10^{-5}	678	978	69.3	0.7	7
6‡	26	10^{-2}	10^{-5}	284	698	40.7	0.08	31	10^{-3}	10^{-5}	617	948	65.1	0.6	8
8§	28	10^{-2}	10^{-5}	1565	841	184.9	0.37	30	10^{-3}	10^{-5}	2067	1064	194.4	1.9	5
8§	31	10^{-2}	10^{-6}	71	187	43.3	0.01	27	10^{-2}	10^{-5}	553	822	67.2	0.1	10
10‖	29	10^{-2}	10^{-5}	1882	1003	187.6	0.37	27	10^{-3}	10^{-5}	1580	989	161.8	1.6	4
19†	30	10^{-3}	10^{-6}	896	435	206.0	0.21	30	10^{-4}	10^{-6}	653	244	275.8	2.8	14
19†	33	10^{-2}	10^{-6}	371	394	93.5	0.02	32	10^{-3}	10^{-6}	866	330	262.4	0.26	13
20†	34	10^{-3}	5×10^{-6}	221	489	45.4	0.23	29	10^{-4}	5×10^{-6}	493	692	71.3	3.6	14

* The figures in this column represent the relative activities of the samples when compared with the controls at the concentrations indicated with *Nicotiana glutinosa* as test plant.

† Ultracentrifugally prepared virus protein used in these experiments.

‡ Duplicates.

§ Inactivated by 5 per cent HCHO at pH 6.0.

‖ " " 5 " " " " " 7.0.

rection factor because of the effect of inactive protein on the lesion count. While this may reduce the accuracy of the values obtained for the activities of such preparations, there can be little doubt that an increase in activity followed reactivation. The differences can also be demonstrated by inoculating one side of the leaves of plants with a preparation before reactivation and the other side with the corresponding preparation after reactivation without further treatment or dilution.

TABLE VII

Reactivation of Inactive or Nearly Inactive Formolized Tobacco Mosaic Virus Protein Preparations

Experiment	Before reactivation			After reactivation		
	No. of leaves*	Concentration (gm. protein/cc.)	Lesions per leaf	No. of leaves*	Concentration (gm. protein/cc.)	Lesions per leaf
5	15	10^{-2}	0.0	15	10^{-2}	0.0
7b	12	0.7×10^{-2}	0.2	15	0.7×10^{-2}	57.5
7b	15	0.7×10^{-3}	0.0	14	0.7×10^{-3}	16.1
12a	15	10^{-2}	0.4	14	10^{-2}	8.0
12a	14	10^{-3}	0.0	14	10^{-3}	1.7
12b	15	10^{-2}	0.0	15	10^{-2}	2.1
12b				14	10^{-3}	0.21
12c	15	10^{-2}	0.0	15	10^{-2}	0.47
12c				15	10^{-3}	0.14
20†	13	0.5×10^{-2}	0.4	15	0.5×10^{-2}	4.0

* *Nicotiana glutinosa* used as test plant.

† Ultracentrifugally prepared virus protein used in this experiment.

The activities of preparations less active than those already considered are difficult to measure accurately, but reactivation of such preparations can be demonstrated by inoculating whole leaves with the preparations before and after reactivation. The results obtained are given in Table VII. It was not found practicable to use higher concentrations than 10^{-2} gm. protein per cubic centimeter. Samples that retained some activity were invariably reactivated to an appreciable extent, but, if the inactivation had proceeded too far, reactivation was not obtained. An intermediate point was found where preparations at 10^{-2} gm./cc. were inactive but were active following the reactivation process. It seems probable, therefore, that two

simultaneous reactions occur, one reversible and the other irreversible. The former reaction probably reaches an equilibrium, while the latter one continues slowly, thus accounting for the long time required for complete inactivation. It was hoped that by varying the conditions the irreversible reaction could be minimized or stopped or the reversible one favored, but so far little success has been attained following the use of different concentrations of reactants, of different pH values, or of different aldehydes.

An increase in concentration of formaldehyde caused an increase in rate of inactivation, while an increase in hydrogen ion concentration to pH 6 resulted in a decrease in the rate of reaction. The results of attempts to reactivate samples that were inactivated under such conditions are included in Tables IV–VI. It may be noted that essentially the same degree of reactivation was obtained with such samples as was obtained in the other cases. When partially inactivated protein solutions were allowed to stand at 3° for several months, their activity increased only slightly. If they were then dialyzed at pH 3 for 3 days, their activity increased approximately 10-fold or to about that which was obtainable if they had been reactivated soon after inactivation.

It is evident that the amount of reactivation that may be secured with formolized tobacco mosaic virus protein is many times greater than can be attributed to experimental error, to the toxic action of inactive protein, or to the removal of any remaining traces of formaldehyde by the prolonged dialysis. It seemed desirable, therefore, to attempt to correlate the inactivation and subsequent reactivation with changes in the protein molecule.

The Effect of Inactivation and of Reactivation on Amino Groups.—Formaldehyde-inactivated tobacco mosaic virus protein has been reported to contain only about 60 per cent of the amino nitrogen (Van Slyke) of active protein (2). This result has been confirmed and in addition it has been found that partially inactivated virus proteins contain intermediate amounts of amino nitrogen. However, because of the small amount of amino nitrogen, it was not possible to differentiate between the amino nitrogen contents of 1 per cent, 0.1 per cent, and 0.0 per cent active protein preparations. It is, therefore, not surprising that no changes were detected when the samples were

reactivated. It is quite possible that part of the formaldehyde is removed by the acetic acid used in the Van Slyke amino nitrogen procedure and the measurements that were made refer only to the protein irreversibly inactivated.

Since Dulière (18) found that formolized proteins did not react with ninhydrin at 37°, the possibility of using this reagent to detect changes in amino nitrogen was investigated. The procedure to be

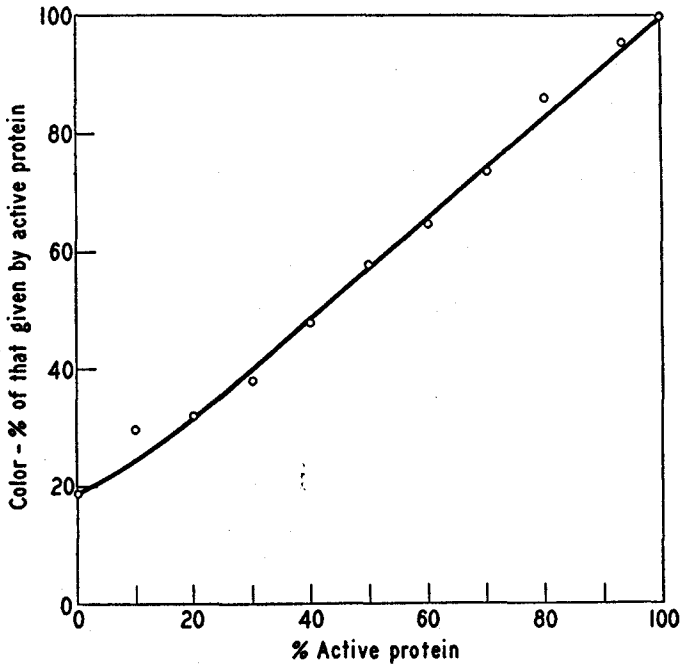

FIG. 2. The relative amounts of color obtained by means of ninhydrin with mixtures of active virus protein and virus protein completely inactivated by formaldehyde.

described was found most satisfactory. To 1 cc. of a dialyzed virus solution (5 to 10 mg. protein per cubic centimeter) in a 3 cc. test tube were added 0.3 cc. pyridine and 0.3 cc. of a 2 per cent aqueous solution of ninhydrin. The solutions were mixed, stoppered, placed in an incubator at 37°C., and at the end of 20 hours they were diluted to 25 cc., allowed to stand ½ hour, and then compared by means of a photoelectric colorimeter. Fig. 2 presents the results of an experiment

in which mixtures consisting of active and of completely inactive virus proteins in different proportions were adjusted to the same total protein concentration and triplicate samples subjected to the above procedure. It may be seen that, considering the fact that the inactive protein gives some color, there is a good proportionality between the amount of color obtained and the proportion of active protein present. There was some variation in results, but changes in chromogenic power of 10 per cent were easily detectable when triplicate samples were used. Observations on the rate at which the samples developed perceptible amounts of color indicated that a given sample became colored in a shorter period of time than one containing 10 per cent less active protein.

Since the ninhydrin test seemed to be useful as a method of determining changes in amino nitrogen, it was used to determine the effect of inactivation and of reactivation on that grouping. In most of the experiments in which this test was used, the preparations were dialyzed at pH 3 for several days before the experiments were started. Partially inactivated preparations were then obtained and a portion of each reactivated as described above. The controls that were used consisted of portions of the original active virus protein preparations that were given exactly the same treatment as the samples, except that formaldehyde was not added. Triplicate samples were then treated with ninhydrin. The results of one of the more uniform experiments are shown in Fig. 3. The color obtained with a given sample is expressed as per cent of that given by the control. Curve A represents the relative color intensities developed by samples following exposure to formaldehyde for the indicated periods of time, dialysis, and treatment with ninhydrin as described. Completely inactive preparations gave a small amount of color after 20 hours of incubation. Curve B represents the color intensity developed by the same samples after reactivation. In this experiment there was an increase in chromogenic power apparently due to the reactivation process. There was also a decrease in the chromogenic power of the control, and, if it can be assumed that the same change occurred in each sample, one may correct Curve B for such changes and thus obtain for the reactivated preparation Curve C. In the case of certain samples, dialysis at pH 3 for 3 days caused an increase in the color obtainable with the

controls. In such instances there were usually still greater changes in the same direction in the samples, thus still indicating an increase in chromogenic power due to the reactivation process. With but few exceptions, the above type of results was obtained. Invariably

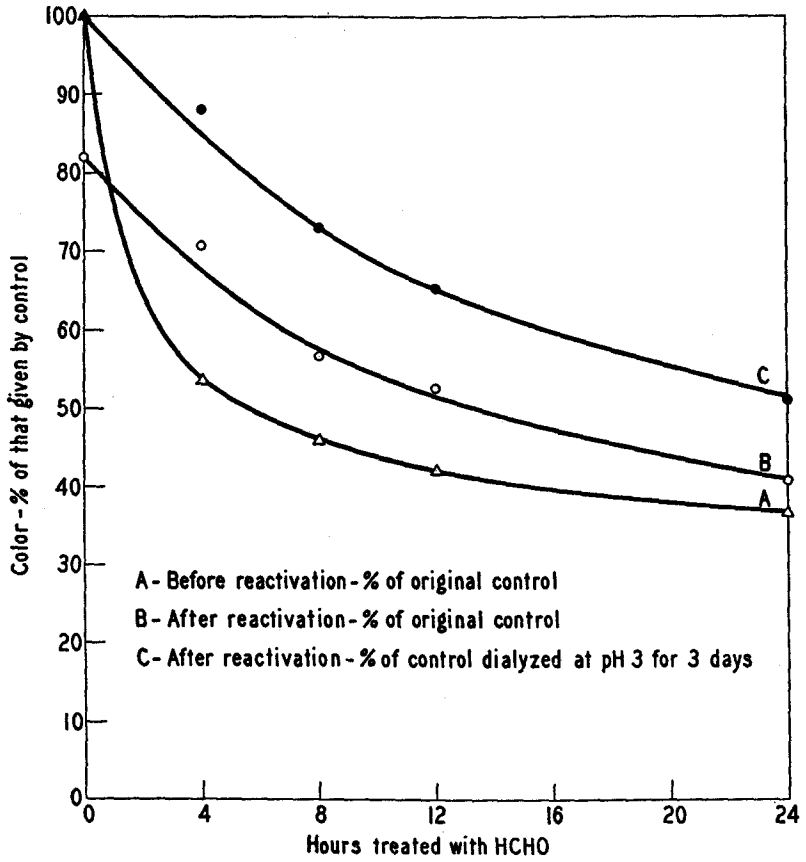

FIG. 3. The effect of inactivation and of reactivation on the amount of color obtainable with ninhydrin. Protein concentration of 5 mg./cc.

a gradual reduction in the amount of color obtainable with ninhydrin accompanied inactivation, but in a few cases, however, there was no indication of any increase due to reactivation.

Considered as a whole, the data indicate that the reactivation process results in an increase in the number of groups that react with

ninhydrin, presumably the amino groups. The variation in behavior of the controls and the occasional occurrence of negative results detract from the significance of the data, but a simultaneous increase in the color developed with ninhydrin and in activity occurred too consistently to be attributed to chance. As there seems to be no quantitative relationship between the amount of reactivation and the increase of color with ninhydrin, the results can only be considered as evidence that the reactivation process results in an increase in the groups that react with ninhydrin and that this is very probably due to the removal of formaldehyde from the amino groups.

Effect of Inactivation and of Reactivation on the Groups that React with Folin's Reagent.—It was found that inactivation of the virus protein was accompanied by a decrease in the number of groups that reacted with Folin's phenol reagent. The effect was most noticeable when the color was allowed to develop at pH 7.5 to 8.0. The method used was essentially that of Herriott (19) except that sodium phosphate was used instead of the potassium salt, thus permitting the use of a stronger buffer without the formation of a precipitate. In a 50 cc. flask were placed 10 cc. of H_2O and 10 cc. of alkaline phosphate buffer (M/2 Na_2HPO_4 + NaOH, pH 10). 3 cc. of Folin's phenol reagent (diluted 1 to 3) were then added, followed immediately by 0.5 cc. of the dialyzed protein solution (5–10 mg./cc.). This sequence was followed so that the protein at no time would be subjected to excessively alkaline or acid conditions, for denatured proteins may give higher values than native ones (20). The resultant reaction was at pH 7.7, varying somewhat with the particular buffer or phenol reagent. After standing at room temperature for 3 hours the samples were compared by means of a photoelectric colorimeter.

The same samples and controls that were used in the ninhydrin tests were also tested with Folin's phenol reagent. Results obtained with the latter reagent were more consistent than those obtained with ninhydrin. In every case a decrease in the color obtainable with Folin's reagent accompanied inactivation, and in only one experiment was there no indication of an increase in the number of groups that react with the reagent due to the reactivation process, and in that particular case very little reactivation was obtained. The reactivation process usually caused a decrease in the chromogenic power of the controls, yet in spite of this the reactivated samples practically always gave more color with the reagent than did the corresponding samples before reactivation. The results of a typical experiment are given in Fig. 4. Curve A represents the color developed when samples exposed to formaldehyde for the indicated periods of time were dialyzed and then tested with Folin's reagent. Curve B represents the color developed by the same samples after reactivation and is based on the same control. When the data are corrected for the effect of dialysis on the control, Curve C is obtained.

The consistency with which the above type of results was obtained indicates that formaldehyde reacts with some group or groups present on the protein molecule that also react with Folin's reagent and that the reaction is partially reversible. As far as it is known, the only

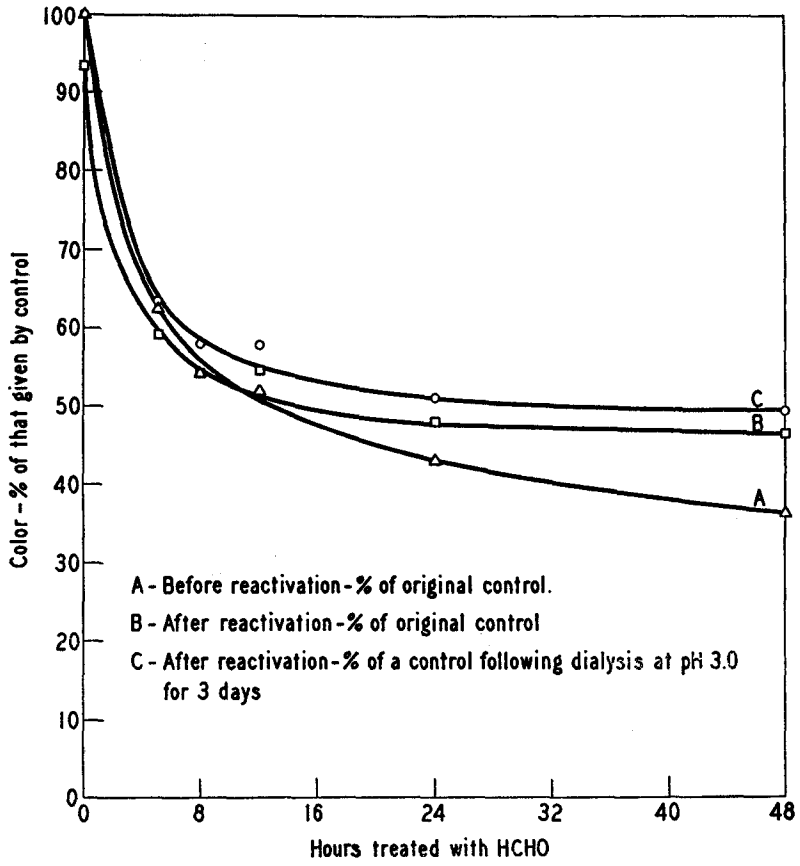

FIG. 4. The effect of inactivation and of reactivation on the amount of color obtainable with Folin's phenol reagent at pH 7.7.

groups in proteins that react with that reagent are the phenolic groups of tyrosine (21), the indole nuclei of tryptophane (22), and sulfhydryl groups (23). Tobacco mosaic virus protein contains no sulfhydryl groups, but does contain appreciable quantities of the other two types of groupings. It, therefore, seemed desirable to determine the effect

of formaldehyde on the latter types when present in simple compounds. Solutions of 0.0025 M tryptophane, tyrosine, glycyltyrosine, glycyltryptophane, and indole propionic acid were treated with 2

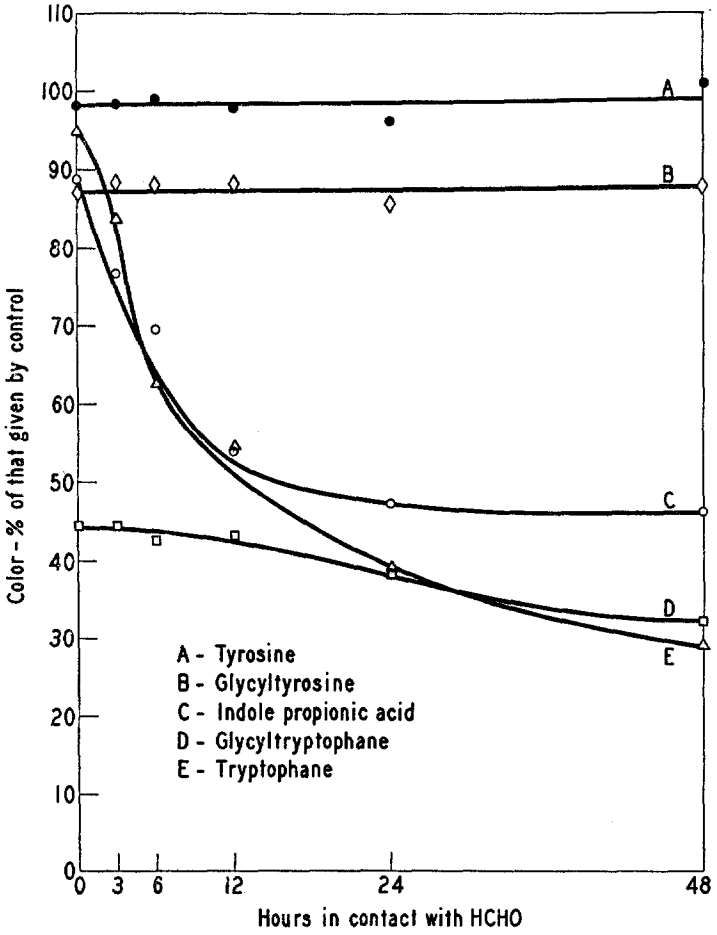

FIG. 5. The effect of 2 per cent HCHO on certain indole derivatives at pH 7. The curves represent the relative amounts of color developed with Folin's phenol reagent at pH 7.7.

per cent formaldehyde in the presence of M/10 phosphate buffer at pH 7. At intervals samples were removed and tested with Folin's reagent, as already described. As formaldehyde gave no color with

the reagent, tests were made in the presence of the excess aldehyde. As may be seen from Fig. 5 tyrosine did not react and glycyltyrosine gave only a small immediate reaction, but in the case of the compounds containing the indole nucleus there was an immediate reaction, especially in the case of the peptide, followed by a gradual decrease in the amount of color obtainable with the reagent. In the case of tryptophane a crystalline precipitate was formed. This is probably 3, 4, 5, 6-tetrahydro-4-carboline-5-carbonic acid, for Wadsworth and Pangborn (5) obtained such a compound under essentially similar conditions. When the crystalline compound was removed and washed, it gave some color with Folin's reagent at pH 7.7 but only a fraction of that obtainable with tryptophane itself. It will be seen from Fig. 4 that no immediate reaction between formaldehyde and the virus protein is recorded. However, it is possible that such a reaction may actually have occurred, since the data for the virus protein were obtained using dialyzed samples, hence it was impossible to obtain a sample representing zero time for the reaction cannot be stopped instantaneously by dialysis. In experiments with the virus protein in which determinations were made without removal of the excess formaldehyde, there was an immediate decrease of about 15 per cent in the amount of color given with Folin's reagent at pH 7.7 At other points along the curve practically the same amount of color was obtained with a given sample, regardless of whether the formaldehyde was present or removed by dialysis. In view of the above results, it seems quite probable that the data obtained with the virus protein are due at least in part to the reaction of formaldehyde with the indole nuclei of the protein.

Other Reactive Groups.—Other groups on the protein molecule that might react with formaldehyde are the imidazole ring of histidine (24) and the guanidine nucleus of arginine (25). Unpublished results obtained in this laboratory indicate that histidine is either absent or occurs in only small amounts in hydrolysates of the virus protein. Furthermore, qualitative tests for histidine on the intact protein molecule gave negative results. It was not deemed necessary, therefore, to study at this time the possible effect of formaldehyde on the imidazole ring. The virus protein was found to give a strong Sakaguchi test for arginine (26) and this test was not diminished by form-

aldehyde treatment. The test was carried out at a fairly strong alkaline reaction, and it may be that under such conditions formaldehyde was removed, although Eaton (25) found that when diphtheria toxin was treated with formaldehyde the Sakaguchi test was diminished. Nothing is known concerning the action of formaldehyde on the virus nucleic acid. However, it is quite probable that nucleic acid is not concerned in the chemical changes that have been measured. The virus nucleic acid reacts slowly with nitrous acid and, under the conditions used, the gas given off by it in the Van Slyke determinations would not be measurable. The virus nucleic acid gave only a trace of color with Folin's reagent at pH 7.7 and could not account for over 1 per cent of that given by the protein. Addition of nucleic acid to formolized virus protein did not affect the color obtainable with ninhydrin.

The Action of Other Aldehydes.—The fact that formaldehyde reacts reversibly with more than one grouping of the virus protein molecule renders it difficult to determine the specific reaction causing loss of virus activity. The action of other aldehydes on the virus protein was investigated, in view of the possibility that some of them might give but one type of reaction. Acetaldehyde, propionaldehyde, and butyraldehyde gave essentially the same qualitative results as did formaldehyde. Each caused a simultaneous decrease in virus activity, in groups that react with ninhydrin, and in groups that react with Folin's phenol reagent. Reactivation was obtained in each case, although in general it was somewhat less than that obtained with formolized virus protein. There was also less indication of chemical changes accompanying reactivation. Benzaldehyde caused a gradual reduction in activity, but there were either no accompanying changes in the number of amino or other groups or such changes were quite small. Samples of virus protein that were partially inactivated by benzaldehyde were reactivated to about half the extent of those that were inactivated by formaldehyde. When a solution of the virus protein was treated with furfural, an insoluble precipitate was formed. Considerable protein remained in solution, but such solutions were quite opalescent. They possessed about 2 per cent of their original activity and gave less color with Folin's reagent or with ninhydrin than did the original active virus protein. Dialysis at pH 3 for 3 days caused about a 10-fold increase in activity in most preparations.

DISCUSSION

The reactivation of formolized virus differs from that which has been secured following inactivation by safranine (27, 28) or with the salts of heavy metals (29, 30), for in the latter instances the complexes are insoluble and the possibility that they are non-infectious because of toxicity has not been excluded. The fact that purified proteins were used and that tests were made in the absence of excess inactivating agent also distinguishes it from the reactivations reported by Zinsser and Seastone (31), Perdrau (32), and Schultz and Gebhardt (7). The results from tests with impure preparations could possibly be due to reactions in which the impurities play an important rôle. Tests for activity in which the excess inactivating agent has not been removed are open to question until it has been shown definitely that the agent has no effect on the susceptibility of the host. It is possible that an impurity present in very small amounts might account for the results obtained with the reactivation of formolized virus, but it seems quite unlikely in view of the constancy of the results, regardless of the method of purification used. Reactivation or recovery of spores or bacteria that have been attenuated with the salts of heavy metals (33–35) or with heat (36) have been reported, but in such cases no correlation has yet been established between chemical changes and changes in biological activity such as those described in this paper. It seems likely that in the case of microorganisms attenuation is due at least in part to an injury that the cell itself is able to overcome or to one that the cell can survive provided the poison is removed within a certain period of time. In the present experiments, however, there is no evidence of an "injury" to the virus due to formaldehyde that the virus itself can overcome. The introduction of the active virus remaining in partially inactivated preparations or of the virus in reactivated preparations into a host results in the production of active virus in a manner and at a rate that is indistinguishable from that following the introduction of ordinary virus. Furthermore, evidence has been presented that the formaldehyde can remain in combination with the virus protein for long periods of time without diminishing its ability to be reactivated. On the other hand, Gegenbauer (33) found that bacteria attenuated with $HgCl_2$ could not be recovered by chemical treatment if they were allowed to remain in the dormant state for too long a period of time. The data reported

here are not regarded as evidence that the virus is either non-living or living but solely as evidence that virus activity is dependent upon a certain chemical structure that is found in the virus protein molecule.

The data presented indicate that the inactivation of tobacco mosaic virus protein by formaldehyde is due to a blocking of active groups or at least to a destruction of a part of the structure essential for virus activity. The chemical reactions taking place may be quite complex. In view of the partial reversibility of the reaction, it appears quite likely that the formaldehyde combines with the protein. If inactivation were the result of a reduction, an oxidation, or a cleavage due to the formaldehyde, it seems quite unlikely that dialysis would bring about a reversal of the reaction. That formaldehyde is actually bound to the protein is indicated by the fact that the formolized proteins give a very strong Rosenheim-Acree test upon the addition of H_2SO_4 and an oxidizing agent. It is possible, however, that the irreversible part of the reaction is due to one of the other types of reactions.

The similarities between the reactions occurring when the virus protein is treated with formaldehyde and those taking place between certain amino acids and formaldehyde under the same conditions are probably not without significance. It was shown that the indole nucleus, even though substituted in a variety of ways, reacted with formaldehyde under the conditions used, and it is reasonable to assume that it reacts with formaldehyde when present in a protein molecule.

The changes that occur in amino nitrogen in the reaction between formaldehyde and virus protein are similar in many respects to those that occur in reactions between formaldehyde and amino acids or other proteins. In the well known formol titration, equilibrium is reached quite rapidly, hence the reaction must differ from that causing inactivation of the virus, although of course the reaction may occur and be readily reversed in the plant. If amino acids or proteins are allowed to react with formaldehyde for several days, a gradual decrease of amino nitrogen (Van Slyke) occurs (4). The rate varies with the amino acid used, as does also the total amount reacting. It has been shown by Wadsworth and Pangborn (5) that this reaction with formaldehyde is partially reversible and that the reversibility decreases as the reaction between the amino acid and formaldehyde proceeds.

On the basis of their results, they suggested that there are at least three stages in the combination of formaldehyde with amino groups: (1) a loosely associated molecular compound, (2) a labile chemical compound, possibly a methylene or hydroxymethyl derivative, and (3) a stable compound, probably formed by further reaction or rearrangement of the labile compound. Stages (1) and (2) were considered to be reversible, whereas stage (3) was not. This sequence of reactions serves to explain the present data very well. Thus, the results on the inactivation and the reactivation and the accompanying chemical changes could be explained by known chemical reactions. It is not possible at present to identify the specific reaction that causes the change in activity, but since these two reactions are associated so closely with the change in activity it seems quite likely that one or the other (or perhaps both) is responsible. It is concluded, therefore, that either the indole nuclei, amino groups, or both are part of the structure necessary for virus activity.

SUMMARY

A marked reactivation of tobacco mosaic virus protein that has been partially or completely inactivated by formaldehyde was obtained by dialysis at pH 3. The activity of partially inactivated virus proteins was generally increased about 10-fold by the reactivation process. It was also found possible to reactivate completely inactive preparations to an appreciable extent. It was shown that the inactivation and the subsequent reactivation cannot be explained by the toxicity of the formaldehyde or of the formolized protein or by aggregation.

Inactivation was accompanied by a decrease in amino groups as indicated by Van Slyke gasometric determinations and by colorimetric estimations using ninhydrin. Inactivation also causes a decrease in the number of groups that react with Folin's reagent at pH 7.7. The latter are probably the indole nuclei of tryptophane, for it was demonstrated that tryptophane, glycyltryptophane, and indole propionic acid react with formaldehyde in a similar manner, while tyrosine and glycyltyrosine do not. Evidence that reactivation is accompanied by an increase in amino nitrogen and in groups that react with Folin's reagent was obtained by colorimetric estimation.

The demonstration that the addition of formaldehyde to the virus protein results in a simultaneous decrease of activity, of amino groups, and of groups that react with Folin's phenol reagent, and that under conditions favorable for the removal of formaldehyde the virus activity is regained and the number of such groups increases, indicates that certain of these groups play at least a partial rôle in the structure necessary for virus activity. These changes can best be interpreted on the basis of known chemical reactions and are considered as evidence that virus activity is a specific property of the protein.

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