REVERSIBLE INACTIVATION OF THE SUBSTANCE INDUCING TRANSFORMATION OF PNEUMOCOCCAL TYPES*

By MACLYN McCARTY, M. D.,

Lieutenant, Medical Corps, United States Naval Reserve

(*From the United States Navy Research Unit at the Hospital of The Rockefeller Institute for Medical Research*)

(Received for publication, March 9, 1945)

Recent studies on the chemical nature of the substance inducing transformation of pneumococcal types have provided strong evidence that this biologically active substance is a nucleic acid of the desoxyribose type (1). Minute amounts of a desoxyribonucleic acid fraction isolated from Type III pneumococci are capable of causing unencapsulated variants of Pneumococcus Type II to acquire the capsular structure and type specificity of Pneumococcus Type III. The results of chemical, enzymatic, and physicochemical analysis of the purified material indicate that it is the nucleic acid itself which induces transformation. Thus, the desoxyribonucleic acid of the pneumococcal cell appears to possess biological specificity, the chemical basis of which is as yet undetermined.

In the course of experiments directed toward isolation of transforming substance from Pneumococcus Type III, it was discovered that treatment of extracts with small amounts of ascorbic acid resulted in complete loss of biological activity. After purified material became available, a further study of this type of inactivation was carried out with respect both to the mechanism of inactivation and to the possibility of obtaining information concerning the chemical basis of the specificity of the transforming substance. Interest in the phenomenon was enhanced by the finding that the inactivation is reversible (2). The present paper deals with the results of a further analysis of this reversible inactivation and is concerned primarily with the mechanism of action of ascorbic acid.

EXPERIMENTAL

Titration of Activity of the Transforming Substance.—The Type III pneumococcal transforming substance was purified by the method previously described (1). The preparation (RC1) used throughout these experiments contained 8.13 per cent phosphorus and 14.46 per cent nitrogen. Transformation was effected with as little as 0.01 microgram.

The method of quantitative titration of transforming activity was given in detail in the paper cited. Serial dilutions of the solution of transforming substance are made in physiological saline, and 0.2 cc. of each dilution is added to quadruplicate tubes containing 2.0 cc. of

* The Bureau of Medicine and Surgery of the U. S. Navy does not necessarily undertake to endorse views and opinions which are expressed in this paper.

a special serum broth. The tubes are inoculated with 0.05 cc. of a 10^{-4} dilution of a young (5 to 8 hour) culture of a strain of R pneumococcus (R36A) derived from Pneumococcus Type II. After 20 to 24 hours at 37°C. all the cultures are plated on blood agar on which the colonies of the transformed Type III cells are readily recognizable. Further identification is carried out by bacteriological and serological tests.

Inactivation of the Transforming Substance by Ascorbic Acid

The addition of ascorbic acid in neutral solution to the active desoxyribonucleic acid fraction of Pneumococcus Type III results in no apparent change in

TABLE I

Inactivation of Transforming Substance by Ascorbic Acid

Solution of transforming substance in 0.05 M phosphate buffer, pH 7.5, treated at 37°C. with:	Transforming activity				
	Dilution*	Quadruplicate tests			
Ascorbic acid 0.01 M for 5 min.	10^{-1}	R only	R only	R only	R only
	10^{-2}	R "	R "	R "	R "
	10^{-3}	R "	R "	R "	R "
	10^{-4}	R "	R "	R "	R "
Ascorbic acid 0.01 M for 20 min.	10^{-1}	R only	R only	R only	R only
	10^{-2}	R "	R "	R "	R "
	10^{-3}	R "	R "	R "	R "
	10^{-4}	R "	R "	R "	R "
Ascorbic acid 0.01 M for 60 min.	10^{-1}	S III	R only	R only	R only
	10^{-2}	R only	R "	R "	R "
	10^{-3}	R "	R "	R "	R "
	10^{-4}	R "	R "	R "	R "
Control. No ascorbic acid	10^{-1}	S III	S III	S III	S III
	10^{-2}	S III	S III	S III	S III
	10^{-3}	S III	S III	S III	S III
	10^{-4}	S III	S III	S III	S III

* Dilution of the transforming substance in saline. Since 0.2 cc. of these dilutions is added to 2.0 cc. of serum broth, the final dilution in the transforming system is approximately tenfold more than that indicated.

the physical properties of the material. The viscosity of the solution is not reduced and upon the addition of alcohol the nucleate precipitates in the characteristic fibrous form. However, as the result of treatment with ascorbic acid the nucleate completely loses its biological activity, as shown in the following experiment.

A 0.02 M solution of ascorbic acid was prepared by dissolving 35.2 mg. of synthetic ascorbic acid (LaRoche) in 2.0 cc. of 0.1 N NaOH and bringing the volume to 10.0 cc. with 0.05 M phosphate buffer, pH 7.5. Because of the relative instability of ascorbic acid at this pH, the solution was used immediately after preparation. The 0.02 M solution was mixed with an

equal volume of a solution of transforming preparation RC1 (approximately 2 mg./cc) in phosphate buffer at pH 7.5, and the mixture was incubated at 37°C. At 5 minutes, 20 minutes, and 1 hour, aliquots of this solution were removed and precipitated by 4 volumes of alcohol. Alcohol precipitation serves the dual purpose of stopping the reaction and sterilizing the mixture prior to testing for transforming activity. The ascorbic acid being soluble in the alcohol is thus separated from the fibrous precipitate of the transforming substance. The precipitates of transforming substance were recovered by centrifugation and redissolved in saline. Serial tenfold dilutions were prepared in saline and tested for transforming activity. Active material without the addition of ascorbic acid was subjected to the same conditions and served as control. The results are recorded in Table I.

In Table I, and in subsequent tables, "S III" indicates the occurrence of transformation as evidenced by the recovery of encapsulated cells of Pneumococcus Type III, while the term "R only" means that transformation has not taken place, and that only the unencapsulated R variants were recovered. In Table I it can be seen that transformation failed to occur in all but one of the tubes containing transforming substance treated with ascorbic acid. On the other hand, in the control series, transformation occurred uniformly in all tubes containing the untreated active material. Thus, in 0.01 M concentration, ascorbic acid was capable of bringing about total inactivation of the transforming agent in as short a period as 5 minutes. This type of result has been obtained repeatedly under the conditions described.

Effect of Cupric Ion on Ascorbic Acid Inactivation

At the alkaline pH used in these experiments, the autoxidation of ascorbic acid proceeds at a relatively rapid rate. Cupric ion is known to exert a marked catalytic effect upon the autoxidation of ascorbic acid, and indeed the rapid rate of the reaction in solutions such as those employed in the above experiment has been ascribed to the presence of minute traces of copper in the distilled water (3). Since it appeared possible that the inactivation of the transforming substance might be related to the oxidation of the ascorbic acid, the effect of added cupric ion was tested.

A 0.002 M solution of ascorbic acid was obtained by making a 1:10 dilution in phosphate buffer of a 0.02 M solution prepared as described in the previous experiment. This solution was mixed with an equal volume of a solution of the transforming substance, with and without the addition of $CuSO_4$ in a final concentration of 0.00001 M. The effect of $CuSO_4$ alone at this concentration was tested as control. After 1 hour at 37°C., the solutions were precipitated by 4 volumes of alcohol, and the precipitates of transforming substance were redissolved in saline and tested as before for transforming activity. The results of this experiment are recorded in Table II.

The data recorded in Table II show that ascorbic acid at 0.001 M concentration without added copper caused only partial loss of activity as indicated by the fact that transformation occurs in half the tubes which received the 10^{-3} dilution of the treated material. In the presence of small amounts of $CuSO_4$,

however, inactivation was complete and no transformation was obtained. The cupric ion by itself did not alter the activity as compared with control material.

The fact that cupric ion markedly enhances the inactivating action of ascorbic acid strongly supports the possibility that this action of ascorbic acid results from autoxidation. Sulfhydryl compounds such as glutathione and cysteine are known to prevent or retard the autoxidation of ascorbic acid, and the effect of this type of compound was investigated.

TABLE II

Effect of Cupric Ion on the Inactivation of Transforming Substance by Ascorbic Acid

Solution of transforming substance in 0.05 M phosphate buffer, pH 7.5, treated 1 hr. at 37°C. with:	Transforming activity				
	Dilution	Quadruplicate tests			
Ascorbic acid 0.001 M + $CuSO_4$ 0.00001 M	10^{-1}	R only	R only	R only	R only
	10^{-2}	R "	R "	R "	R "
	10^{-3}	R "	R "	R "	R "
	10^{-4}	R "	R "	R "	R "
Ascorbic acid 0.001 M	10^{-1}	S III	S III	S III	S III
	10^{-2}	S III	S III	S III	S III
	10^{-3}	R only	S III	S III	R only
	10^{-4}	R "	R only	R only	R "
$CuSO_4$ 0.00001 M	10^{-1}	S III	S III	S III	S III
	10^{-2}	S III	S III	S III	S III
	10^{-3}	S III	S III	S III	S III
	10^{-4}	S III	S III	S III	S III
Untreated control	10^{-1}	S III	S III	S III	S III
	10^{-2}	S III	S III	S III	S III
	10^{-3}	S III	S III	S III	S III
	10^{-4}	S III	S III	S III	S III

The Protective Action of Glutathione

The effect of 0.01 M ascorbic acid was tested on a solution of transforming substance in the presence of 0.01 M glutathione and in the presence of 0.001 M glutathione.

As in the previous experiments, the reaction was carried out for 1 hour at 37°C. in 0.05 M phosphate buffer at pH 7.5. The reaction was stopped by precipitation with 4 volumes of alcohol, the alcohol precipitates redissolved in saline, and serial tenfold dilutions tested for transforming activity. The results are recorded in Table III.

As in the first experiment, treatment with 0.01 M ascorbic acid resulted in complete loss of transforming activity. In the presence of 0.01 M or 0.001 M glutathione, however, this effect was completely inhibited during the 1 hour

incubation at 37°C. and the activity of the transforming substance treated with the combined agents corresponded well with that of the untreated control material.

Cysteine and thioglycolic acid have a protective action similar to that of glutathione. Thus, additional evidence for the rôle of autoxidation in inactivation of the transforming substance is obtained, and it further becomes apparent that although ascorbic acid is primarily a reducing agent, its action on the

TABLE III

Inactivation of Transforming Substance by Ascorbic Acid
Protective Action of Glutathione

Solution of transforming substance in 0.05 M phosphate buffer, pH 7.5, treated 1 hr. at 37°C. with:	Transforming activity				
	Dilution	Quadruplicate tests			
Ascorbic acid 0.01 M	10^{-1}	R only	R only	R only	R only
	10^{-2}	R "	R "	R "	R "
	10^{-3}	R "	R "	R "	R "
	10^{-4}	R "	R "	R "	R "
Ascorbic acid 0.01 M + Glutathione 0.01M	10^{-1}	S III	S III	S III	S III
	10^{-2}	S III	S III	S III	S III
	10^{-3}	S III	S III	S III	S III
	10^{-4}	R only	S III	S III	R only
	10^{-5}	R "	R only	R only	S III
Ascorbic acid 0.01 M + Glutathione 0.001M	10^{-1}	S III	S III	S III	S III
	10^{-2}	S III	S III	S III	S III
	10^{-3}	S III	S III	S III	S III
	10^{-4}	R only	R only	S III	R only
	10^{-5}	R "	R "	R only	R "
Untreated control	10^{-1}	S III	S III	S III	S III
	10^{-2}	S III	S III	S III	S III
	10^{-3}	S III	S III	S III	S III
	10^{-4}	R only	S III	S III	S III
	10^{-5}	R "	R only	R only	R only

transforming substance is probably oxidative in character. In view of this fact, the possible reversal of the inactivation by the use of reducing agents was investigated.

Reversal of Inactivation by Glutathione

A solution of transforming substance was treated with 0.01 M ascorbic acid in 0.05 M phosphate buffer at pH 7.5 for 1 hour at 37°C. as in the preceding experiments. After precipitation by alcohol to remove excess ascorbic acid, the transforming substance was redissolved in saline and the solution was divided into two equal parts. The first part was held as a control of the

completeness of ascorbic acid inactivation. The second portion was mixed with glutathione at a final concentration of 0.02 M and incubated for 1 hour at 37°C. Serial tenfold dilutions of each solution were made and tested for transforming activity in parallel with untreated control material. The results of the transformation tests are given in Table IV.

It can be seen from Table IV that as in the preceding experiments the loss of activity of transforming substance treated with 0.01 M ascorbic acid was complete. However, when an aliquot of this inactivated material was treated with glutathione, there was almost quantitative restoration of the original activity, and the titer was only slightly less than that of untreated transforming substance.

TABLE IV

Inactivation of Transforming Substance by Ascorbic Acid
Reversal of Inactivation by Glutathione

Solution of transforming substance in 0.05 M phosphate buffer, pH 7.5, c.f. treated as follows:	Transforming activity				
	Dilution	Quadruplicate tests			
Ascorbic acid 0.01 M 1 hr. at 37°C.	10^{-1}	R only	R only	R only	R only
	10^{-2}	R "	R "	R "	R "
	10^{-3}	R "	R "	R "	R "
	10^{-4}	R "	R "	R "	R "
Aliquot of ascorbic acid–inactivated material subsequently treated with: Glutathione 0.02 M 1 hr. at 37°C.	10^{-1}	S III	S III	S III	S III
	10^{-2}	S III	S III	S III	S III
	10^{-3}	S III	S III	S III	S III
	10^{-4}	R only	S III	R only	S III
	10^{-5}	S III	R only	R "	R only
Untreated control	10^{-1}	S III	S III	S III	S III
	10^{-2}	S III	S III	S III	S III
	10^{-3}	S III	S III	S III	S III
	10^{-4}	S III	S III	S III	S III
	10^{-5}	S III	S III	R only	R only

The same type of reactivation, though generally less complete, has been achieved with cysteine and thioglycolic acid. It should also be mentioned that reactivation by glutathione is not always so quantitative as in the experiment described above, suggesting that under certain conditions the inactivation may be carried to an irreversible stage.

The action of sulfhydryl compounds in reversing the inactivation by ascorbic acid could be interpreted as evidence in favor of the view that sulfhydryl groups are present in the molecule of the transforming substance, and that it is these groups which are reversibly oxidized and reduced. There is, however, no evidence for the presence of sulfhydryl groups in the purified transforming prepara-

tions. The fact that the nitroprusside reaction is negative, and the further fact that the material suffers no loss of biological activity when treated with iodoacetic acid, make it seem unlikely that reversible inactivation involves sulfhydryl groups in the transforming substance.

The Effect of Other Agents on the Transforming Substance

Since the effect of ascorbic acid is apparently oxidative in nature, the action of certain biological oxidants on the transforming substance was tested. Cytochrome C, α-tocopherol phosphate, flavine phosphate, alloxan, and ferricyanide had no effect on the transforming activity when tested under conditions comparable to those employed in the ascorbic acid experiments. On the other hand, glucoascorbic and isoascorbic acids, substances which possess the characteristic dienol configuration of ascorbic acid but have little or no vitamin C activity, were found to be in all respects similar to ascorbic acid in their effect on the transforming substance. Furthermore, catechol, which may be considered to possess a dienol configuration analogous to that of ascorbic acid, also inactivated the transforming substance when tested under appropriate conditions. In the absence of added cupric ion, catechol at 0.01 M concentration proved to have a very slight effect on transforming activity, but when as little as 10^{-5} M $CuSO_4$ was present, inactivation was almost complete. This effect of catechol was prevented and reversed by sulfhydryl compounds. Its action was therefore completely analogous to that of ascorbic acid.

In the hope of gaining some clue as to the mechanism of ascorbic acid inactivation of the pneumococcal transforming substance, the survey was extended to include other compounds related to catechol. *o*-Aminophenol has properties identical to those of catechol, and in the presence of cupric ion, caused complete inactivation of the transforming substance. Hydroquinone and phenanthrene hydroquinone were also highly effective, and again the action of these compounds was catalyzed by copper and prevented and reversed by sulfhydryl compounds. It is of interest that the oxidized forms of the two latter compounds—*p*-quinone and phenanthrene quinone respectively—exerted only a slight or partial inactivating effect when tested in the same concentration in the presence of copper. Thus, it is apparently not the end-product of oxidation of the hydroquinones but some intermediate substance which is responsible for the action on the transforming substance. *p*-Phenylendiamine and several methyl substituted derivatives of this compound, all of which are oxidized to the same end-product as hydroquinone, *i.e.* *p*-quinone, were also highly active. The action of the *p*-phenylenediamines was also inhibited and reversed by sulfhydryl compounds.[1]

[1] The author is grateful to Dr. L. Michaelis and Dr. S. Granick for supplying many of the compounds used in this study, and expresses his appreciation for their suggestions and advice.

Thus, a group of compounds, all of which undergo a similar type of autoxidation, are capable in the course of oxidation of bringing about a change in the molecule of the transforming substance which results in loss of biological activity.

Effect of Exclusion of Oxygen

Although it seems apparent from the foregoing experiments that inactivation of the transforming substance is the result of oxidation, the rôle of molecular oxygen in the reaction has not been clarified. The effect of exclusion of oxygen was tested by carrying out the reaction between the inactivating agent and th transforming substance under anaerobic conditions. For this purpose, tetra

TABLE V

Effect of Exclusion of Oxygen on Inactivation of Transforming Substance

Solution of transforming substance in 0.05 M phosphate buffer, pH 7.5, treated 1 hr. at 37°C. with:	Transforming activity				
	Dilution	Quadruplicate tests			
0.01 M tetramethyl *p*-phenylene diamine *Aerobically*	10^{-1}	R only	R only	R only	R only
	10^{-2}	R "	R "	R "	R "
	10^{-3}	R "	R "	R "	R "
	10^{-4}	R "	R "	R "	R "
0.01 M tetramethyl *p*-phenylene diamine *Anaerobically*	10^{-1}	S III	S III	S III	S III
	10^{-2}	S III	S III	S III	S III
	10^{-3}	S III	S III	S III	S III
	10^{-4}	R only	R only	S III	S III
Untreated control	10^{-1}	S III	S III	S III	S III
	10^{-2}	S III	S III	S III	S III
	10^{-3}	S III	S III	S III	S III
	10^{-4}	S III	S III	S III	R only

methyl-*p*-phenylenediamine was chosen as the inactivating agent, since it forms a highly colored intermediate oxidation product which serves as an indicator of the effectiveness of exclusion of oxygen.

0.5 cc. of a solution of the transforming substance containing 2×10^{-5} M $CuSO_4$ was placed in a Thunberg tube. 2.4 mg. of tetramethyl-*p*-phenylenediamine dihydrochloride were placed in the side arm. To the dry reagent were added 0.3 cc. 0.05 M phosphate buffer, pH 7.5, and 0.2 cc. 0.1 N NaOH (giving a 0.02 M solution of the reagent), and the tube was immediately evacuated by means of a Hyvac pump. The reagent and transforming substance were then thoroughly mixed and the evacuated tube was incubated for 1 hour at 37°C. The solution remained practically colorless, indicating that effective anaerobiosis had been obtained. The reaction was stopped by the addition of 4 volumes of alcohol. A control experiment was carried out simultaneously with the same concentration of tetramethyl-*p*-phenylenediamine under the usual aerobic conditions. The results of the titration, of transforming activity are recorded in Table V.

Table V shows that under aerobic conditions tetramethyl-*p*-phenylenediamine caused the expected complete inactivation of the transforming substance. When the reaction was carried out in the evacuated Thunberg tube, however, the transforming activity of the treated material was not significantly different from that of the untreated control material. Similar results were obtained in experiments using ascorbic acid and hydroquinone as the inactivating substance.

The Rôle of Hydrogen Peroxide

It is apparent from the foregoing that molecular oxygen is required in the inactivating process. This together with the fact that hydrogen peroxide is known to be formed in the course of autoxidation of ascorbic acid, catechol,

TABLE VI

Effect of Catalase on the Inactivation of Transforming Substance by Ascorbic Acid

Solution of transforming substance in 0.05 M phosphate buffer, pH 7.5, treated 1 hr. at 37°C. with:	Transforming activity				
	Dilution	Quadruplicate tests			
Ascorbic acid 0.01 M	10^{-1}	R only	R only	R only	R only
	10^{-2}	R "	R "	R "	R "
	10^{-3}	R "	R "	R "	R "
	10^{-4}	R "	R "	R "	R "
Ascorbic acid 0.01 M + Catalase 0.01 mg/cc.	10^{-1}	S III	S III	S III	S III
	10^{-2}	S III	S III	S III	S III
	10^{-3}	S III	S III	R only	S III
	10^{-4}	R only	S III	R "	R only
Untreated control	10^{-1}	S III	S III	S III	S III
	10^{-2}	S III	S III	S III	S III
	10^{-3}	S III	R only	S III	S III
	10^{-4}	R only	R "	R only	R only

hydroquinone, etc., suggested the possibility that peroxide might play an important rôle in the inactivation of the transforming substance. This possibility was tested in two ways: (1) indirectly, by the use of catalase to prevent the accumulation of peroxide, and (2) directly, by testing the effect of preformed hydrogen peroxide on the transforming substance.

A preparation of crystalline catalase was obtained through the courtesy of Dr. Michaelis and Dr. S. Granick. 0.5 cc. of a solution of the transforming substance containing 2×10^{-5} M $CuSO_4$ and 10 micrograms of crystalline catalase was mixed with 0.5 cc. of a 0.02 M ascorbic acid solution. A similar tube in which the catalase was omitted was used as control of the activity of the ascorbic acid, and a third tube in which the ascorbic acid was replaced by buffer served as a control of the potency of the transforming substance. After 1 hour at 37°C. the material in each tube was precipitated by alcohol, the precipitate redissolved in saline, and tested as usual for transforming activity. The results are recorded in Table VI.

It can be seen from Table VI that a minute amount of crystalline catalase was sufficient to protect the transforming substance from the inactivating effect of ascorbic acid. This type of experiment leaves little doubt that peroxide is in some way concerned with the inactivation of the transforming substance. It had been previously observed in the case of crude preparations of the transforming substance that hydrogen peroxide caused loss of biological activity, but only when it was used in relatively high concentrations. The effect of

TABLE VII

Effect of Hydrogen Peroxide on the Transforming Substance

Solution of transforming substance in 0.05 M phosphate buffer, pH 7.5, treated at 37°C. with:	Transforming activity				
	Dilution	Quadruplicate tests			
H_2O_2 0.01 M	10^{-1}	S III	R only	S III	R only
	10^{-2}	R only	R "	R only	R "
	10^{-3}	R "	R "	R "	R "
	10^{-4}	R "	R "	R "	R "
H_2O_2 0.003 M	10^{-1}	S III	S III	S III	S III
	10^{-2}	S III	S III	S III	S III
	10^{-3}	R only	R only	R only	R only
	10^{-4}	R "	R "	R "	R "
H_2O_2 0.001 M	10^{-1}	S III	S III	S III	S III
	10^{-2}	S III	S III	S III	S III
	10^{-3}	S III	S III	S III	S III
	10^{-4}	S III	R only	R only	R only
Control—no H_2O_2	10^{-1}	S III	S III	S III	S III
	10^{-2}	S III	S III	S III	S III
	10^{-3}	S III	S III	S III	S III
	10^{-4}	R only	S III	S III	S III

peroxide was reinvestigated, using the purified preparations of transforming substance.

Superoxol (Merck) with a minimum H_2O_2 concentration of 29 per cent was used as the source of peroxide. Dilutions of this concentrated solution were prepared in water at 0°C., the final dilutions being made in cold 0.05 M phosphate buffer, pH 7.5. 0.5 cc. portions of the transforming substance in buffer were mixed with 0.5 cc. portions of the following peroxide solutions: 0.02 M, 0.006 M, and 0.002 M. Thus, the effect of peroxide was tested at final concentrations of 0.01 M, 0.003 M, and 0.001 M. The mixtures were incubated at 37°C. for 1 hour and the reaction stopped by precipitation with alcohol. The alcohol precipitates were redissolved in saline and the solutions were titered for transforming activity with the results recorded in Table VII.

The data in Table VII show that 0.01 M H_2O_2 caused almost complete inactivation, while the 0.003 M concentration brought about partial inactivation and the 0.001 M concentration had only a very slight effect. These findings are typical of the experiments with preformed peroxide. To bring about an inactivating effect comparable to that achieved with ascorbic acid and related compounds, it was necessary to use a considerably higher concentration of hydrogen peroxide than is liberated in the course of autoxidation of the minimally effective amount of ascorbic acid. Thus, while 0.001 M ascorbic acid in the presence of traces of $CuSO_4$ caused complete inactivation, the addition of 0.001 M H_2O_2 to the transforming substance had little effect. Even with ten times this amount of peroxide inactivation was not complete. It is possible that the instability of hydrogen peroxide, and the consequent difficulty of controlling accurately the amount added, was responsible for this discrepancy, but there is another possible interpretation of these findings.

In a study of the inactivation of the enzyme, ascorbic acid oxidase, Powers and Dawson (4) have recently presented good evidence that it is not the hydrogen peroxide formed as a reaction product of the enzymatic oxidation of ascorbic acid which inactivates the enzyme, despite the fact that catalase and peroxidase have a marked protective action. They have suggested that some precursor of hydrogen peroxide is formed which is suceptible not only to catalase but also to peroxidase and hemin, and that it is this precursor which is responsible for the inactivation of the enzyme. It is possible that a similar intermediate compound is formed on autoxidation of ascorbic acid and that this hypothetical intermediate causes the inactivation of the transforming substance. The relatively low inactivating effect of preformed hydrogen peroxide would thus be accounted for.

DISCUSSION

The experimental data presented indicate that a group of chemical substances which are related to one another in that they all undergo a similar type of autoxidation, in each case catalyzed by cupric ion, are capable of causing reversible inactivation of the transforming substance isolated from Pneumococcus Type III. A study of the mechanism of action of these compounds has led to the conclusion that they act by the liberation of peroxide or a peroxide precursor which in turn oxidizes certain unidentified groups in the biologically active molecule with resultant loss of activity. The reversal of this effect with restoration of biological activity can be achieved by the use of sulfhydryl compounds.

Although no evidence has been obtained concerning the identity of the chemical groups in the molecule of transforming substance which are affected in the process of inactivation, the results are of interest in view of the nature of the

active substance. This material as isolated from Pneumococcus Type III appears to be desoxyribonucleic acid in a highly polymerized form, a type of substance not generally considered to possess biological specificity. The experiments reported in this paper indicate that certain groups, presumably not sulfhydryl in character, are susceptible to oxidation and that when these groups are in the oxidized state the substance loses its biological activity. Attempts to demonstrate any chemical or physical alterations in the pneumococcal nucleic acid after oxidative treatment have so far not met with success. For example, preliminary studies indicate that no variation in the characteristic ultraviolet adsorption spectrum results from treatment with ascorbic acid. In addition, the oxidized material is depolymerized by the specific enzyme, desoxyribonuclease, at the same rate as is the native substance.

The effect of ascorbic acid on the activity of the transforming substance is by no means a unique phenomenon. In the past ten years a large number of communications have appeared describing the inactivating effect of ascorbic acid on a variety of biologically active agents, including bacterial toxins, viruses, and enzymes. In those instances in which the mechanism of action of ascorbic acid has been studied in detail, the conclusions are in accord with those reached in the present paper, and it is suggested that inactivation is brought about by a similar process in the case of each of the various biologically active agents.

For example, Lojkin (5) in a study of the action of ascorbic acid on purified preparations of the tobacco mosaic virus, showed that inactivation of the virus occurred in the course of autoxidation of the ascorbic acid. The effect was catalyzed by cupric ion and completely inhibited by exclusion of oxygen. Catalase protected the virus from inactivation and the attention of the author was directed to the possibility that an active peroxide formed during the course of autoxidation was responsible for the inactivating effect. However, both hydrogen peroxide and copper peroxide were much less effective than ascorbic acid in causing inactivations of the virus, and Lojkin concluded that some other form of peroxide is the active agent. The results of Lojkin are therefore strikingly parallel with those reported in the present paper. Incidentally, hydroquinone was shown to have an effect on the tobacco mosaic virus similar to that of ascorbic acid.

Jungeblut (6), in investigating the inactivation of diphtheria toxin by ascorbic acid, was led to suggest that in this case also peroxide is responsible for the effect. Hydrogen peroxide was shown to exert a detoxifying effect, but it can be calculated from Jungeblut's data that, as in the case of the pneumococcal transforming substance and tobacco mosaic virus, considerably more hydrogen peroxide is required to inactivate the toxin than is formed during autoxidation of the minimally effective concentration of ascorbic acid. The evidence therefore indicates that H_2O_2 is not the form of peroxide responsible for inactivation of the diphtheria toxin. The study of Willison (7) has confirmed the oxidative

nature of the action of ascorbic acid on diphtheria toxin, and this writer demonstrated that exclusion of oxygen completely nullified the inactivating effect.

Giri (8) and his coworkers have made a study of the inhibiting action of ascorbic acid on a variety of enzymes. Again the action is recognized as being oxidative in character on the basis of copper-catalyzed autoxidation of ascorbic acid. At present, Giri apparently attributes the effect to intermediate oxidation products involving copper, such as Cu_2O (9).

It is probable, then, that the inactivating effect of ascorbic acid depends upon the same mechanism in the case of substances as diverse as diphtheria toxin, tobacco mosaic virus, several enzymes, and the pneumococcal transforming substance. The protective action of sulfhydryl compounds, and other compounds which inhibit the autoxidation of ascorbic acid, has been observed in many of these studies. However, reversal of the inactivation as described for the pneumococcal transforming substance in the present paper is not so well known. Hanes (10) has observed that inhibition of β-malt amylase by ascorbic acid, and other dienol compounds can be reversed by subsequent treatment of the enzyme-ascorbic acid mixture with sulfhydryl compounds. This observation would appear to be analogous to the reversal of inactivation of the transforming substance. The analogy is carried further by the fact that inhibition of β-amylase by ascorbic acid when catalyzed by added copper is more rapid and less readily reversible, suggesting that the inhibition may be carried to an irreversible stage.

It is not certain at present whether oxidative inactivation of the transforming substance assumes practical importance in the complex biological system in which transformation occurs. Pneumococcal cells are known to produce hydrogen peroxide which accumulates in the medium during growth under aerobic conditions, and since transformation occurs only in the presence of cells which are actively growing, the peroxide formed is a potential threat to the transforming substance. This type of inactivation may be one of the factors responsible for the variability that is sometimes encountered in titrations of transforming activity.

SUMMARY

1. The transforming substance of Pneumococcus Type III is inactivated by treatment with ascorbic acid. This effect of ascorbic acid is catalyzed by traces of cupric ion and is prevented by the presence of sulfhydryl compounds.

2. Under certain conditions, the activity of transforming substance treated with ascorbic acid can be restored by the use of glutathione and other sulfhydryl compounds.

3. Other compounds, such as catechol, hydroquinone, and *p*-phenylenediamine, which undergo autoxidation similar to that of ascorbic acid, have an analogous effect on the transforming substance.

4. The effect of these compounds on the transforming substance is nullified by exclusion of oxygen or by the use of catalase.

5. It is concluded that inactivation of the transforming substance is probably oxidative in character and depends on the formation of peroxides in the course of autoxidation of ascorbic acid or related compounds.

6. The relation of this phenomenon to that of the inactivation of other biologically active substances by ascorbic acid is discussed.

BIBLIOGRAPHY

1. Avery, O. T., MacLeod, C. M., and McCarty, M., *J. Exp. Med.*, 1944, **79,** 137.
2. McCarty, M., *J. Clin. Inv.*, 1944, **23,** 942 (abstract).
3. Barron, E. S. G., DeMeio, R. H., and Klemperer, F., *J. Biol. Chem.*, 1936, **112,** 625.
4. Powers, W. H., and Dawson, C. R., *J. Gen. Physiol.*, 1944, **27,** 181.
5. Lojkin, M., *Contrib. Boyce Thompson Inst.*, 1937, **8,** 445.
6. Jungeblut, C. W., *J. Infect. Dis.*, 1941, **69,** 70.
7. Willison, F. E., *J. Immunol.*, 1943, **47,** 409.
8. Giri, K. V., *Z. physiol. Chem.*, 1938, **254,** 126. Seshagiri Rao, P., and Giri, K. V., *Proc. Indian Acad. Sc.*, 1942, **16,** 190.
9. Giri, K. V., and Seshagiri Rao, P., *Nature*, 1944, **153,** 253.
10. Hanes, C. S., *Biochem. J.*, 1935, **29,** 2588.