STUDIES ON THE CHEMICAL NATURE OF THE SUBSTANCE INDUCING TRANSFORMATION OF PNEUMOCOCCAL TYPES

II. Effect of Desoxyribonuclease on the Biological Activity of the Transforming Substance*

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The substance inducing transformation of pneumococcal types has been isolated from Pneumococcus Type III in the form of a desoxyribonucleic acid fraction (1). The data obtained by chemical, enzymatic, and serological analysis, as well as by electrophoresis and ultracentrifugation of the purified material strongly suggest that the nucleic acid is itself responsible for the biological activity.

The enzymatic analysis was begun early in the course of the attempts to determine the nature of the transforming substance. Relatively unpurified pneumococcal extracts were subjected to enzymatic study in the hope that by this approach some clue might be obtained as to the identity of the biologically active constituent. Crystalline trypsin, chymotrypsin, and ribonuclease had no effect on the transforming substance, but it was found that certain crude enzyme preparations were able to bring about complete loss of transforming activity. When the possible importance of desoxyribonucleic acid was suggested by chemical fractionation, the experiments with crude enzyme preparations were extended to determine whether their ability to destroy the activity of the transforming principle could be correlated with any enzymatic action on authentic samples of desoxyribonucleic acid of non-bacterial origin. A variety of crude enzymes were tested both for their ability to inactivate the transforming substance, and for their effect on desoxyribonucleic acid from animal tissues (1). Of the enzyme preparations tested only those capable of depolymerizing authentic desoxyribonucleic acid were effective in destroying the transforming activity of pneumococcal extracts. Other parallelisms between the two actions were observed: for example, sodium fluoride inhibited both the depolymerizing action and the inactivation of the transforming sub-

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stance. Thus, indirect evidence was obtained by enzymatic analysis in support of the thesis that the active transforming substance is a desoxyribonucleic acid (1).

Since no purified preparation of desoxyribonuclease was available, purification of the enzyme was undertaken in this laboratory in order that the enzymatic evidence concerning the nature of the transforming substance could be made more direct and conclusive. The enzyme has been isolated from beef pancreas in highly active form relatively free from other enzymes, and its properties have been investigated in some detail (2). It is the purpose of the present paper to describe the action of purified desoxyribonuclease on the biological activity of the transforming substance of Pneumococcus Type III.

EXPERIMENTAL

Effect of Citrate Inhibition of the Enzyme.—It has been found that desoxyribonuclease from beef pancreas, as well as from all other sources studied, requires the presence of a metallic activator (2). Magnesium ion appears to be the naturally occurring activator, although manganese at the same molar concentration is equally effective. It has been shown that sodium citrate is a potent inhibitor of magnesium-activated desoxyribonuclease, presumably because citrate forms a soluble complex with magnesium, and thus prevents activation of the enzyme. On the other hand, the manganese-activated enzyme is not appreciably inhibited by the presence of citrate. Thus citrate inhibition is dependent upon the nature of the metallic activator (2). In order to determine whether citrate inhibits the action of desoxyribonuclease on the transforming principle, and whether a similar relationship exists with respect to the nature of the metallic activator, the following experiment was carried out.

A 2 mg. per cc. solution of purified transforming substance isolated from Pneumococcus Type III (RCI) was prepared in 0.025 M veronal buffer, pH 7.5. To two 0.5 cc. portions of this solution 0.1 cc. of 0.03 M $MgSO_4$ was added. To one of these tubes 0.1 cc. of 0.1 M sodium citrate was also added. Two additional tubes were prepared containing the same amount of transforming substance, but using instead of $MgSO_4$ a similar amount and concentration of $MnSO_4$. As in the first series, citrate was added to one of these tubes. The volume of each of the four tubes was brought to 0.9 cc. with veronal buffer, pH 7.5, and 0.1 cc. of a solution of partially purified desoxyribonuclease containing 0.001 mg. of protein was added to all tubes. Thus, the final concentration of Mg^{++} and Mn^{++} was 0.003 M, and the final concentration of citrate 0.01 M. The enzymatic reaction was allowed to proceed at 30°C. for 15 minutes, and then was stopped by heating all tubes at 60°C. for 15 minutes. Serial tenfold dilutions of the reaction mixtures were then made and tested for transforming activity according to the method of titration previously described (1). 0.2 cc. of each dilution was added to quadruplicate tubes containing 2.0 cc. each of special serum broth. The tubes were inoculated with 0.05 cc. of a 10^{-4} dilution of a broth culture of R cells (R36A) derived from Pneumococcus Type II. After 20 hours' incubation at 37°C., each culture was plated on blood agar, and the presence of Type III cells was determined by bacteriological and serological tests. The results are recorded in Table I.

It will be seen from the results presented in Table I that in the presence of either 0.003 M $MgSO_4$ or 0.003 M $MnSO_4$, the enzyme caused complete inactivation of the transforming substance. However, 0.01 M sodium citrate brought about almost complete inhibition of the magnesium-activated enzyme,

TABLE I

Citrate Inhibition of the Action of Desoxyribonuclease on the Transforming Substance Effect of the Nature of the Metallic Activator

Transforming substance treated with:	Transforming activity				
	Dilution of enzyme-treated material	Quadruplicate tests			
		1	2	3	4
Mg^{++} activated enzyme	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 "
Mn^{++} activated enzyme	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 "
Mg^{++} activated enzyme + sodium citrate	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	R only
Mn^{++} activated enzyme + sodium citrate	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 "
No enzyme (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

* "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 only unencapsulated R variants were recovered.

and the titer of the transforming substance after treatment with the enzyme in the presence of citrate and magnesium was only slightly lower than that of the control material. On the other hand, citrate had no apparent effect on the manganese-activated enzyme, and the transforming substance was completely destroyed.

The above results parallel those obtained in a study of the effect of citrate

on the action of the enzyme on desoxyribonucleic acid from calf thymus (2). The data emphasize the close relationship between the ability of the enzyme preparation to inactivate the transforming substance on the one hand, and to depolymerize desoxyribonucleic acid on the other. It therefore appears that the same enzymatic process is involved in both instances, since in each case the conditions of activation and inhibition of the enzyme are identical.

Titration of the Activity of Purified Desoxyribonuclease.—Purified preparations of desoxyribonuclease from beef pancreas have no demonstrable phosphatase, lipase, or ribonuclease activity (2). Traces of a proteolytic enzyme are present, but in order to detect proteolytic activity an enzyme concentration of more than 0.2 mg. per cc. must be used, while a definite measurable effect on the viscosity of sodium desoxyribonucleate solutions can be demonstrated at concentrations of less than 0.01 *microgram* per cc. (2). Thus, in terms of enzymatic activity the proteolytic enzyme is a minor contaminant and does not complicate the interpretation of results obtained with appropriate concentrations of desoxyribonuclease.

In the following titration of the activity of the enzyme on the transforming substance, the concentrations of the various components in the reaction system were identical with those used in the standard method for measuring the activity of the enzyme on calf thymus nucleic acid (2). However, the total volume of the reaction system was smaller, in order to conserve the available supply of the pneumococcal nucleate. Consequently, it was not feasible to measure quantitatively the fall in viscosity of the solution of transforming substance.

A 5 mg. per cc. solution of the dried, purified enzyme (preparation 16) was prepared in water. To obtain an appropriate concentration for the activity titration, a 10,000- fold dilution (containing 0.5 μg per cc. of enzyme protein) of this enzyme solution was prepared in volumetric flasks. Further twofold dilutions were made and used in the test. Since it has been shown that gelatin retards denaturation of the enzyme in dilute solution (2), the diluting fluid consisted of an aqueous solution of 0.25 per cent gelatin and 0.075 M $MgSO_4$.

1.2 cc. samples of a 0.1 per cent solution of purified Type III transforming substance (RCI) in 0.025 M veronal buffer, pH 7.5, were introduced into each of five tubes. 0.05 cc. of the falling twofold dilutions of enzyme was added to the first four tubes. The dilutions of the enzyme used contained 0.25, 0.125, 0.062, and 0.031 μg. per cc. so that the final enzyme concentrations in the reaction system were 0.01, 0.005, 0.0025, and 0.00125 μg. per cc. respectively. 0.05 cc. of the gelatin-$MgSO_4$ diluent alone was added to the fifth tube which served as control.

The enzyme was allowed to act at 30°C. for 30 minutes after which the tubes were heated in a water bath at 60°C. for 10 minutes to stop the reaction. Serial tenfold dilutions of the reaction mixtures were then made in saline, and tested for transforming activity by the usual procedure. The results are recorded in Table II.

It can be seen from Table II that the enzyme in concentrations of 0.01 and 0.005 μg. per cc. completely destroyed the activity of the transforming agent

in the 30 minute period. Inactivation was practically complete when 0.0025 μg. per cc. of enzyme was used, and even with the smallest concentration of the enzyme (0.00125 μg. per cc.) more than a 90 per cent reduction in the activity of the transforming substance resulted.

It is apparent, then, that the purified desoxyribonuclease is exceedingly active in destroying the pneumococcal transforming substance. It is of in-

TABLE II

The Action of Desoxyribonuclease on the Transforming Substance
Titration of Activity of Purified Enzyme

Concentration of enzyme in reaction system	Transforming activity				
	Dilution of enzyme-treated material	Quadruplicate tests			
		1	2	3	4
0.01 μg. per cc.	10^{-1}	R only*	R only	R only	R only
	10^{-2}	R "	R "	R "	R "
	10^{-3}	R "	R "	R "	R "
0.005 μg. per cc.	10^{-1}	R only	R only	R only	R only
	10^{-2}	R "	R "	R "	R "
	10^{-3}	R "	R "	R "	R "
0.0025 μg. per cc.	10^{-1}	R only	R only	S III*	R only
	10^{-2}	R "	R "	R only	R "
	10^{-3}	R "	R "	R "	R "
0.00125 μg. per cc.	10^{-1}	S III	S III	S III	S III
	10^{-2}	R only	S III	S III	S III
	10^{-3}	R "	R only	R only	R only
	10^{-4}	R "	R "	R "	R "
None (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	R only	R only	R only

* Symbols same as in Table I.

terest that the titration reveals almost the same end-point as that obtained when the activity of the identical preparation is measured by the viscosimetric method on calf thymus desoxyribonucleic acid (2). The fall in viscosity of animal nucleate solution in the presence of 0.00125 μg. per cc. of enzyme, the smallest amount used in the above test, is slow but measurable, and approximately a 10 per cent decrease in viscosity occurs in 30 minutes under the conditions of the test. For reasons stated above, the effect on viscosity was not measured quantitatively in the experiments using the transforming substance

as substrate. However, the qualitative effect was readily recognizable, and in those tubes in which complete enzymatic destruction of the transforming activity had occurred, there was a corresponding loss in viscosity when compared with the control tube containing no enzyme.

DISCUSSION

The fact that a purified preparation of desoxyribonuclease in exceedingly low concentration is capable of destroying irreversibly the Type III transforming substance provides strong confirmatory evidence for the view that biological activity is a property of the desoxyribonucleic acid. In this connection it is of interest that irreversible inactivation of the transforming agent by enzymatic digestion differs from that form of inactivation brought about by ascorbic acid and certain related compounds, since in the latter instance, the reaction is reversible and full activity can be restored by the use of sulfhydryl compounds (3).

The possibility has been recognized that the activity of the transforming agent might be referable to minute amounts of some other substance such as protein in the purified preparations. The results of the present investigation show that in order to detect proteolytic activity, it is necessary to use an amount of purified desoxyribonuclease 100,000 times greater than that required to cause rapid and complete destruction of activity of the transforming substance. This evidence, in conjunction with the data previously reported on the chemical and physical properties of the active principle, leaves little doubt that the ability of a pneumococcal extract to induce transformation depends upon the presence of a highly polymerized and specific form of desoxyribonucleic acid, and that this constituent is the fundamental unit of the transforming principle.

The objection can be raised that the nucleic acid may merely serve as a "carrier" for some hypothetical substance, presumably protein, which possesses the specific transforming activity. Depolymerization of the nucleic acid would according to this hypothesis, destroy the effectiveness of the essential carrier and thus result in loss of biological activity. There is no evidence in favor of such a hypothesis, and it is supported chiefly by the traditional view that nucleic acids are devoid of biological specificity. On the contrary, there are indications that even minor disruptions in the long-chain nucleic acid molecule have a profound effect on biological activity. Thus, treatment of the transforming substance with concentrations of desoxyribonuclease so small that only a slight fall in viscosity occurs causes a marked loss of biological activity. It is suggested that the initial stages of enzymatic depolymerization which are reflected only by minimal changes in the physical properties of the nucleate are sufficient to bring about destruction of specific activity.

Although the results of enzymatic studies provide additional evidence for

the specific rôle of desoxyribonucleic acid in pneumococcal transformation, they throw no light on the possible chemical basis for this specificity. It remains one of the challenging problems for future research to determine what sort of configurational or structural differences can be demonstrated between desoxyribonucleates of separate specificities. In this connection, it should be pointed out that in all probability only a relatively small number of the total molecules in an active preparation of desoxyribonucleate from Pneumococcus Type III are capable of inducing transformation. This is suggested by the fact that extraction of unencapsulated R pneumococci yields a similar desoxyribonucleate fraction which at present can be distinguished from the Type III material only by the fact that the former is inactive in the transforming system. It is possible that the nucleic acid of the R pneumococcus is concerned with innumerable other functions of the bacterial cell, in a way similar to that in which capsular development is controlled by the transforming substance. The desoxyribonucleic acid from Type III pneumococci would then necessarily comprise not only molecules endowed with transforming activity, but in addition a variety of others which determine the structure and metabolic activities possessed in common by both the encapsulated (S) and unencapsulated (R) forms. If these considerations have any foundation in fact, the task of discovering the chemical basis of biological specificity of desoxyribonucleic acids becomes extremely complex, since a given preparation will represent a mixture of a large number of entities of diverse specificity.

An example of a roughly analogous situation is afforded by the gamma globulin fraction of immune sera. In this instance, although the preparation is chemically and physically homogeneous, it has been shown by immunological techniques to consist of a variety of antibody molecules of diverse specificity. Determination of differences in specificity of these various protein molecules which are chemically indistinguishable one from another is made possible only by the use of corresponding specific antigens, selectively reactive with homologous antibody protein.

In the case of desoxyribonucleic acid, the techniques of pneumococcal transformation provide at the present time the only available method for determining differences in selective activities and specificities of this biologically important group of chemical substances.

SUMMARY

It has been shown that extremely minute amounts of purified preparations of desoxyribonuclease are capable of bringing about the complete and irreversible inactivation of the transforming substance of Pneumococcus Type III. The significance of the effect of the enzyme, and its bearing on the chemical nature of the transforming substance, together with certain considerations

concerning the biological specificity of desoxyribonucleic acids in general, are discussed.

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