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

III. An Improved Method for the Isolation of the Transforming Substance and Its Application to Pneumococcus Types II, III, and VI*

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In the first paper of this series a method was described for obtaining the transforming substance of Pneumococcus Type III in the form of a desoxyribonucleic acid fraction (1). The yield of active material was not high, and for this reason a more efficient procedure has been devised which is based upon recently acquired knowledge concerning the properties of the transforming substance and the enzyme that destroys it. In addition, it has been possible to adapt the new method to the isolation and purification of the transforming substance from pneumococci other than those of Type III and to show that in this case also the active substance is contained in a desoxyribonucleic acid fraction.

EXPERIMENTAL

Preparation of Transforming Substance from Pneumococcus Type III.—In earlier work it was found necessary to heat-kill the bacterial cells at 65°C. immediately after they had been collected by centrifugation (1). This step was required because of the presence in the pneumococcal cell of an enzyme which destroys the activity of the transforming substance. Consequently, if early inactivation of this enzyme was neglected, the biological activity of the extract was greatly impaired or completely lost. On the other hand, cells heated at a temperature known to inactivate the enzyme are so altered that complete extraction of the active material is difficult as shown by the fact that the residual Type III cells after extraction by the method previously outlined still contain an appreciable amount of the active desoxyribonucleic acid fraction.

Evidence has accumulated which indicates that the pneumococcal enzyme

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responsible for the inactivation of the transforming substance is a desoxyribonuclease which attacks the highly polymerized form of desoxyribonucleic acid (1–3). Although no purified preparation of pneumococcal desoxyribonuclease has been obtained, its enzymatic behavior appears to be analogous to that of the desoxyribonuclease prepared from beef pancreas, the properties of which have been described in a separate communication (2). For example, the heat lability, optimum pH, and dependence upon activation by magnesium ion (or manganese ion) are the same for the pneumococcal enzyme as for that obtained from the pancreas. It has been shown that the enzyme from both these sources has little, if any, activity in the absence of magnesium ions, and that citrate acts as an effective inhibitor of desoxyribonuclease by virtue of its capacity to form a soluble complex with magnesium. Citrate inhibition of the pneumococcal enzyme forms the basis of the method to be described.

The presence of citrate in sufficient concentration to inhibit desoxyribonuclease completely has no retarding action on autolysis of living pneumococcal cells. The effect of bile salts in promoting rapid lysis seems to be accelerated rather than retarded. Thus, by allowing lysis to proceed in the presence of desoxycholate and citrate, it is possible to release quickly the transforming substance from living cells and at the same time to preserve its full activity by inhibiting the destructive action of the enzyme. The procedure for obtaining the active material in purified form from the lysate is described in the following section.

Preparative Method.—The cells from 50 liters of a 12 hour culture of Pneumococcus Type III are collected in a steam-driven Sharples centrifuge as previously described (1). The packed cells are resuspended in 500 cc. of a solution containing 0.1 M sodium chloride and 0.1 M sodium citrate. 5.0 cc. of 10 per cent solution of sodium desoxycholate are added. Lysis begins almost immediately and within a few minutes the creamy suspension is changed into a viscous, translucent solution and no formed elements can be found in films prepared and stained by the Gram technique. After 30 minutes at room temperature, one-third volume of chloroform and one-tenth volume of amyl alcohol are added to the lysate and the mixture is shaken mechanically for ½ hour. The material is centrifuged and the supernatant fluid is separated by pipetting from the chloroform emulsion. Shaking with chloroform is repeated two additional times after which the extract is quite clear and greenish-yellow in color. The addition to the extract of two volumes of alcohol with stirring results in the formation of two distinct types of precipitate, one finely granular, and the other in the form of a heavy mass of fibrous material which rapidly falls to the bottom. The less rapidly sedimenting granular precipitate is immediately decanted and the compact fibrous precipitate which contains, among other constituents, the active principle is removed and washed with alcohol.

A considerable amount of active material is entrained in the chloroform-protein gel and can be recovered in large part by extracting the combined gels with 300 cc. of the citrate-saline solution. After centrifugation this extract is also precipitated by alcohol and the fibrous precipitate is combined with that already obtained.

The combined fibrous precipitates are dissolved in 300 cc. saline and the solution is shaken twice with chloroform and amyl alcohol. The solution is now clear and colorless. Two volumes of alcohol are added and the fibrous precipitate formed is again lifted out, washed

with alcohol, and redissolved in 100 to 150 cc. of 0.85 per cent NaCl. 5 mg. of the purified bacterial enzyme capable of hydrolyzing the Type III capsular polysaccharide (4) and 10 mg. of crystalline ribonuclease are added. Digestion is carried out in a cellophane sac during dialysis at 37°C. against a solution consisting of equal parts of saline and 0.05 M veronal buffer, pH 7.8. The decomposition of the capsular polysaccharide is followed by serological tests, as previously described (1). After digestion has proceeded for 6 to 8 hours at 37°C., the material is placed in the refrigerator and dialysis is continued in the cold overnight to complete the removal of the dialyzable end-products of enzymatic action. The solution is mixed with two volumes of alcohol and the fibrous precipitate thus obtained is redissolved in 75 to 100 cc. of saline. Final deproteinization is effected by 2 to 3 successive treatments with chloroform and amyl alcohol in order to remove the added enzyme protein and any remaining traces of pneumococcal protein.

At this point the partially purified material contains a desoxyribonucleic acid and a serologically active substance which has been identified as the somatic C polysaccharide. The C polysaccharide is apparently in a more highly polymerized form than that encountered in the original procedure, and the alcoholic fractionation method previously described does not separate it from the desoxyribonucleic acid. However, use has been made of the fact that in the presence of calcium, desoxyribonucleic acid is precipitable by small amounts of alcohol, while the C polysaccharide remains soluble under these conditions. Thus, an almost quantitative separation as measured by serological techniques is readily effected. The procedure is as follows:—

After final deproteinization, as described above, one-tenth volume of 10 per cent $CaCl_2$ is added to the saline solution of partially purified transforming substance. Upon the addition of two-tenths volume of alcohol, the desoxyribonucleic acid is precipitated and with it all the transforming activity. The fibrous precipitate is washed in saline containing $CaCl_2$ and alcohol in the same concentration as the precipitating mixture, and redissolved in saline. Precipitation by calcium and alcohol is repeated to remove the last traces of serologically active C polysaccharide. The fibrous precipitate is redissolved in saline and preserved by storage in the cold.

Properties of the Purified Material.—The properties of the desoxyribonucleic acid thus prepared are the same as those reported previously for preparations obtained from heat-killed cells (1). Solutions are highly viscous, give a strong diphenylamine test for desoxyribose, and have a nitrogen and phosphorus content characteristic of nucleic acid. The material is serologically inactive when tested with high titer Type III anti-pneumococcal serum, and gives negative qualitative tests for ribonucleic acid and protein. The transforming activity is of the same order as that of preparations obtained by the earlier method, and less than 0.01 μg. suffices to induce transformation.

The chief advantage of the method is that the yield of purified material is markedly increased. Thus, 60 to 80 mg. are recovered from 50 liters of culture, representing a yield fivefold greater than that obtained by the previous method.

Isolation of Transforming Substance from Pneumococci of Types Other Than Type III

Attempts to obtain the transforming substance from pneumococci of Types I, II, and XIV by the extraction of heat-killed cells have yielded only relatively

impure and weakly active extracts. It appeared likely, therefore, that lysis of living pneumococci in the presence of citrate would yield larger amounts of active material and afford a better opportunity of purifying the transforming substance.

A second difficulty encountered in attempts to isolate the transforming material from other types, in a degree of purity comparable to that of the Type III substance, was the fact that no method was available for separating the capsular polysaccharide from the active material. In the case of Type III, the soluble bacterial enzyme which hydrolyzes the capsular polysaccharide served as a very efficient tool for this purpose. However, since enzymes in purified form capable of decomposing the capsular polysaccharide of other types of pneumococci are not available, the separation of these carbohydrates from the active desoxyribonucleic acid fraction can be achieved only by chemical means. It has now been found that precipitation with calcium and alcohol, as described in the preceding section for the removal of the somatic C polysaccharide, can also be used in the same way to remove the capsular polysaccharides of certain types. For example, in the presence of calcium the capsular polysaccharides of Pneumococcus Types II and VI are not precipitated by low concentrations of alcohol which throw down the desoxyribonucleic acid fraction, and in these instances the transforming substance can be separated from the carbohydrate by this technique. On the other hand, with certain other types (*e.g.* Types III and XIV), the calcium salt of the capsular polysaccharide precipitates with the desoxyribonucleic acid fraction and no separation can be achieved by this means.

By using the procedures mentioned, *i.e.* lysis of living cells in the presence of citrate and separation of the polysaccharides with calcium and alcohol, the transforming substance has been prepared from Type II pneumococci in a state of purity comparable to that of the Type III material.

Preparation of the Transforming Substance from Pneumococcus Type II.—A stock strain of Pneumococcus Type II (D39) was used as source material. The cells from 50 liters of an 8 hour culture are collected and the initial procedures involving the use of citrate in preparing an active lysate of the living cells and the technique of deproteinizing the crude material thus obtained are carried out by the method described above for preparing the Type III substance. Since no purified preparation of the enzyme capable of decomposing the Type II capsular polysaccharide is available, enzymatic digestion of the extract with ribonuclease alone is carried out under the same conditions as those just described in the use of the combined enzymes in the Type III extract. This procedure facilitates further purification, since the split products of ribonucleic acid are much more readily separable from the desoxyribonucleic acid fraction than is the intact ribonucleic acid. After enzymatic treatment the material is shaken one or more additional times with chloroform and amyl alcohol to complete the deproteinization. Two volumes of alcohol are then added to the protein-free solution, and the resulting fibrous precipitate is redissolved in 80 cc. saline.

The solution of partially purified material is made up chiefly of desoxyribonucleic acid, Type II capsular polysaccharide, and the somatic C polysaccharide. In order to separate

the nucleic acid from these carbohydrates the calcium-alcohol method is employed as follows: Upon the addition of one-tenth volume of 10 per cent $CaCl_2$ and two-tenths volume of ethyl alcohol, a fibrous precipitate is formed which consists of the desoxyribonucleic acid. This precipitate is washed in saline containing 1 per cent $CaCl_2$ and 20 per cent alcohol, redissolved in saline, and further purified by precipitating a second time with calcium and alcohol. This final precipitate is redissolved in saline. As indicated by the diphenylamine test, all of the desoxyribonucleic acid is present in the calcium-alcohol precipitate. The properties of the desoxyribonucleic acid fraction will be defined below.

Effectiveness of the Calcium-Alcohol Method of Fractionation.—The material remaining in the supernatant fluid after removal of the calcium-alcohol precipitate was recovered by adding excess of alcohol. The fraction thus obtained was redissolved in the same volume of saline as that used in the solution of the desoxyribonucleic acid fraction, and as will be shown consisted largely of the capsular polysaccharide and somatic C carbohydrate. In order to demonstrate the sharp separation achieved by the use of calcium-alcohol fractionation, both of these fractions were tested for serological properties and transforming activity. Serial dilutions of the two fractions were made in saline and precipitin reactions using Type II anti-pneumococcus horse serum were carried out by the techniques described in previous communications from this laboratory. The results are presented in Table I.

It can be seen from the results of precipitin reactions presented in Table I that the material soluble in two-tenths volume of alcohol in the presence of $CaCl_2$ reacted with immune serum when the solution was diluted 1:1000, whereas in the same antiserum the calcium-alcohol insoluble fraction gave only a questionable reaction in the lowest dilution tested. From the results of the precipitin tests it is evident, therefore, that a sharp and almost quantitative separation of the desoxyribonucleic acid fraction from the serologically active polysaccharides is effected by calcium-alcohol fractionation.

Tests of the biological activity of these same fractions in the transforming system were carried out by the method previously described. The results of the transformation tests are shown in Table II.

From the data recorded in Table II it is evident that the fraction containing the serologically active material possessed no transforming activity even at the highest concentration used On the other hand, the desoxyribonucleic acid fraction was active in a 10^{-3} dilution.[1] The transforming tests in conjunction with the serological data emphasize the effectiveness of the chemical procedure used in removing the polysaccharide fraction from the active transforming substance.

It should be noted that the above experiment is not, strictly speaking, an example of pneumococcus transformation since both the extract and the R strain were derived from Pneumococcus Type II. This fact, however, does not alter the significance of the results, since this particular R strain, which has been under continuous cultivation and study in this laboratory during the past several years, has never been observed to revert spontaneously to its

[1] Since 0.2 cc. of this dilution was added to 2 cc. of serum broth the final dilution in the transforming system was approximately tenfold greater than that indicated.

original specific type. Indeed, all attempts to cause it to revert by repeated animal passage and by serial subcultures in anti-R serum have invariably failed. Moreover, reversion can be induced, as in the present instance, only when the specific transforming substance is used.

TABLE I

Serological Tests with Fractions Obtained by Precipitating with 0.2 Volume of Alcohol in the Presence of Calcium Ion

Fraction	Initial dilution of fraction					
	1:3	1:10	1:30	1:100	1:300	1:1000
Precipitate at 0.2 volume of alcohol (Desoxyribonucleic acid)	Very faint trace	−	−	−	−	−
Material soluble at 0.2 volume of alcohol	++++	+++±	+++	++	+	±

++++, pronounced precipitate with clear supernate.
−, no precipitate.

TABLE II

Test of Transforming Activity of Fractions of Type II Extract Obtained by Precipitating with 0.2 Volume of Alcohol in Presence of Calcium Ion

Fraction	Transforming activity				
	Dilution*	Quadruplicate tests			
Precipitate at 0.2 volume of alcohol (desoxyribonucleic acid)	10^{-1}	S II	S II	S II	S II
	10^{-2}	S II	S II	S II	S II
	10^{-3}	S II	R only	S II	R only
	10^{-4}	R only	R only	R only	R only
Material soluble at 0.2 volume of alcohol (serologically active)	10^{-1}	R only	R only	R only	R only
	10^{-2}	R "	R "	R "	R "

Tubes in which only the R variant was recovered are designated as "R only." S II indicates the occurrence of encapsulated Type II cells.
* Dilution of fraction in saline. See footnote 1.

Properties of the Type II Transforming Substance.—The general properties of the purified Type II material are in all respects identical with those of the Type III transforming substance, and the two can be differentiated only on the basis of the specificity of their biological action. Qualitative chemical tests for protein are negative, and the material gives no appreciable reaction with high titer Type II antisera.

Elementary chemical analysis reveals a nitrogen content of 15.76 per cent and phosphorus content of 8.50 per cent. The nitrogen-phosphorus ratio is

thus 1.85 which is slightly higher than that of most samples of the Type III transforming substance. However, the figures fall well within the range of variation encountered among different samples of desoxyribonucleic acid prepared from the same source by a single method.

Without any modification in preparative procedures, the method outlined for obtaining the Type II transforming substance has also been successfully applied to the isolation of a biologically active desoxyribonucleic acid fraction from Pneumococcus Type VI.

DISCUSSION

It is of interest that a study of the properties of the enzyme which inactivates the transforming substance has led to a more efficient method of obtaining the purified material from the pneumococcal cells. Although it is possible to inactivate the pneumococcal enzyme by heating the bacterial suspension, extraction of the active material from heat-killed cells is difficult and incomplete. On the other hand, when release of the active substance is accomplished by lysis of the living cells, some loss of activity results from enzymatic action even when the suspension is rapidly lysed in the cold and then immediately heated as in the procedure described by Alloway (5). The finding that the enzyme in question is activated by magnesium and is effectively inhibited by citrate afforded a means of obtaining the transforming substance from autolyzed cells without the use of heat.

The effect of calcium on the alcohol precipitability of desoxyribonucleic acid has been used in the final purification of the active material. In the presence of excess calcium ion the active desoxyribonucleic acid is completely precipitated by as little as two-tenths volume of alcohol. The highly polymeric pneumococcal polysaccharides have physical properties similar to desoxyribonucleic acid and thus are difficult to separate from it. However, it has been shown that the precipitability of certain of the polysaccharides is not affected by the presence of excess calcium, and sharp separation from the desoxyribonucleic acid can be achieved.

Thus, in addition to increasing the yield of purified transforming substance, the application of the method outlined has provided a means of isolating the substance from pneumococci other than those of Type III. As in the case of the Type III material, the Type II transforming substance has been found to be associated with the desoxyribonucleic acid fraction. The results are thus confirmatory of the previous studies on the chemical nature of the transforming principle and serve to emphasize that in the instances studied, as in probably all other types of pneumococci, the active agents belong to the same general class of chemical substances. Although the individual desoxyribonucleates of different types cannot yet be distinguished from one another on chemical grounds, the selective specificity they exhibit in inducing transforma-

tion is difficult to interpret save in terms of individual differences in chemical structure and molecular configuration.

SUMMARY

1. An improved method is outlined for the isolation and purification of the pneumococcal transforming substance. This method makes use of the fact that citrate inhibits the destructive action of the enzyme, desoxyribonuclease, which is released together with the active material during lysis of the living bacterial cells. A fivefold greater yield of purified transforming agent is obtained by the present method than by the procedure previously described.

2. The specific transforming substance has been isolated from pneumococci of types II and VI, in addition to Type III. In each instance the biologically active material has been found to consist of desoxyribonucleic acid.

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