CHEMO-IMMUNOLOGICAL STUDIES ON CONJUGATED CARBOHYDRATE-PROTEINS

IV. The Synthesis of the *p*-Aminobenzyl Ether of the Soluble Specific Substance of Type III Pneumococcus and Its Coupling with Protein

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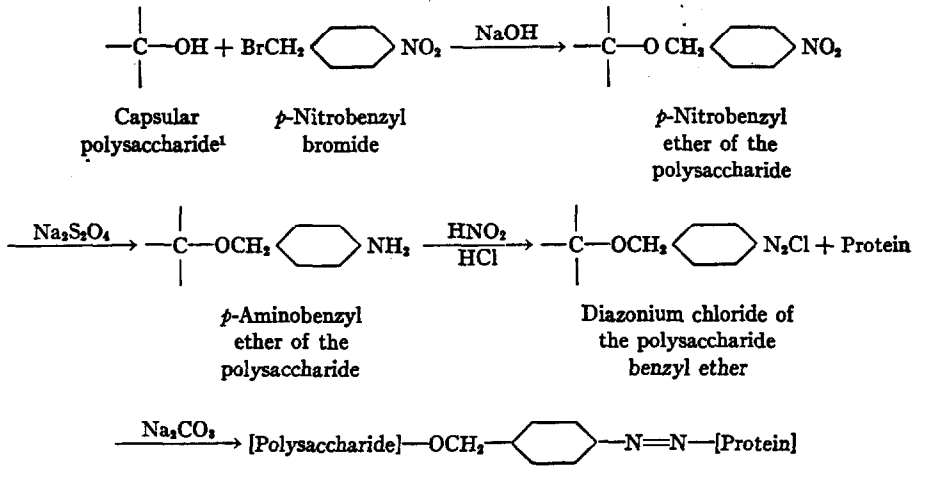
The immunological rôle of specific polysaccharides from encapsulated microorganisms has been discussed in previous communications from this laboratory (1). The capsular carbohydrate of Type III Pneumococcus may be regarded as a pure chemical entity, a polysaccharide free from nitrogenous impurities, retaining the immunological property of reacting specifically in high dilutions with homologous antiserum, yet incapable of inciting the formation of type-specific immune bodies when injected into rabbits. It has been shown, however, that simple carbohydrates when combined with proteins (2) can give rise to specific antibodies. The specificity of the antibodies thus induced is dependent upon the chemical constitution of the carbohydrate irrespective of the protein to which it is bound.

It occurred to us that if the pneumococcus polysaccharide could be combined with a foreign protein it should be possible to produce a conjoined carbohydrate-protein antigen capable of stimulating the formation of type-specific pneumococcus antibodies in the animal body. Provided the specificity of the original carbohydrate has not been too greatly altered either through chemical manipulation, or through the introduction of new molecular groupings, one should obtain on immunization with such a "synthetic antigen," antibodies which would be identical in specific action with those produced by immunization with the intact bacterial cells.

The capsular polysaccharide of Type III Pneumococcus has been shown to be a polymer of an aldobionic acid (3). The carbohydrate

itself has an acid equivalent of 338, *i.e.* it has one free carboxyl (COOH) group for every two sugar molecules. In addition to the free carboxyl group there are three free hydroxyl groups (OH) per unit of aldobionic acid in the polymeric form. If the hydrogen atom of one of these three hydroxyl groups can be replaced by a nitrobenzyl group without incurring a loss in specificity of the polysaccharide, the nitro derivative could be reduced to the amino compound which in turn might be coupled through its diazonium derivative to a protein, yielding a conjoined carbohydrate-protein. Such a complex would have only one constituent common to the pneumococcus cell, namely, the capsular polysaccharide.

The synthesis may be diagrammatically represented by the following series of reactions:



In the following account a description is given of the experimental procedure used in the chemical synthesis of the *p*-nitro and *p*-aminobenzyl ethers of the specific carbohydrate of Type III Pneumococcus

[1] Since it is impossible to draw a complete formula for the capsular polysaccharide, the latter has been represented by drawing the one carbon atom bearing a free hydroxyl group, which enters into chemical reaction with *p*-nitrobenzyl bromide in the presence of sodium hydroxide.

and the coupling of the amino derivative to serum globulin. The immunological specificity of this conjoined carbohydrate-protein complex is discussed in an accompanying publication.

EXPERIMENTAL

1. Preparation of the p-Nitrobenzyl Ether of Pneumococcus Type III Soluble Specific Substance

The nitrobenzyl ether of the carbohydrate was prepared by a method similar to that employed by Gomberg (4) in the preparation of the benzyl ethers of various carbohydrate derivatives.

1 gm. of the nitrogen-free Type III pneumococcus polysaccharide was suspended in 35 cc. of water and brought into solution by neutralizing with N/1 sodium hydroxide. To the solution was added 4.7 gm. of finely pulverized *p*-nitrobenzyl bromide. The mixture was heated to 100°C. and vigorously stirred. The nitrobenzyl bromide melted to form an oil which could be fairly well emulsified in the solution by violent stirring. 2.9 cc. of 30 per cent sodium hydroxide were added drop by drop. The rate of addition of the alkali was such that at no time were there more than a few drops in excess. The reaction was complete after ½ hour of heating and stirring, *i.e.*, the theoretical quantity of alkali had been added to neutralize the hydrobromic acid from the *p*-nitrobenzyl bromide, leaving a neutral solution. The contents of the tube was now distilled with steam to rid the reaction mixture of *p*-nitrobenzyl alcohol. The product remaining in the flask, a yellow paste, was cooled to 0°C. and was acidified by the addition of hydrochloric acid. The product was filtered on a hardened paper, washed with small portions of ice water, and finally was dried in a vacuum desiccator.

The dry material was pulverized in a mortar and extracted with acetone in a Soxhlet extractor. A quantity of soluble colored material,—probably condensation products from the *p*-nitrobenzyl bromide, was thus eliminated. The polysaccharide-ether remaining in the extraction thimble was dissolved in alkali, made up to 200 cc. with water, cooled to 0°C., and acidified by the addition of hydrochloric acid. The precipitate (the nitrobenzyl ether of the soluble specific substance) was centrifuged and then reprecipitated. The final product was filtered on a hardened paper and was then dried in a desiccator. About 1.1 gm. of material were recovered.

The nitrobenzyl ether of the soluble specific substance of Pneumococcus was thus obtained as a pale yellow compound, soluble in dilute alkali by virtue of the free carboxyl group in the molecule. The compound itself is insoluble in water, though its sodium salt is readily soluble. The compound may be precipitated by adding acid to an

aqueous solution of its sodium salt. The ether does not dissolve in the usual organic solvents. It is soluble in 75 per cent aqueous acetone, and is slightly soluble in 50 per cent alcohol.

The compound has a specific optical rotation of $-26.50°$, an acid equivalent of 480 (the calculated value for a mononitrobenzyl ether of a polymer of the aldobionic acid $C_{11}H_{19}O_{10}COOH$ = 473), and a nitrogen content of 2.99 per cent (calculated value = 2.96 per cent). The substance reacts specifically with Type III antipneumococcus serum in dilutions of 1:5,000,000.

2. *Preparation of the Aminobenzyl Ether of the Soluble Specific Substance of Type III Pneumococcus*

1 gm. of the nitrobenzyl ether of the soluble substance was suspended in 20 cc. of water. The substance was brought into solution by neutralization with 20 per cent sodium hydroxide. The solution was warmed to 50°C. and to it was slowly added a freshly prepared saturated solution of sodium hydrosulfite. The solution was kept near the neutral point after each addition of hydrosulfite, by the cautious addition of alkali. When a slight excess of the reducing agent persisted, as determined by the ability of the reaction mixture to bleach litmus paper, the solution was cooled in a freezing mixture and then carefully acidified with hydrochloric acid (sp. gr. 1.09). A yellow precipitate of the amino polysaccharide-ether separated out. After centrifugation, it was dissolved, and the solution was dialyzed against successive changes of distilled water until free of chlorides and sulfates. The solution was then poured into 25 volumes of chilled acetone, and after it had flocked out of solution, the amino ether was filtered on a hardened paper. About 0.8 gm. was recovered.

The aminobenzyl ether of the Type III soluble specific substance was obtained as a yellow ash-free amorphous powder soluble in warm water, and in aqueous acetone (75 per cent). It has an optical rotation of $-28.5°$, an acid equivalent of 453 (calculated = 443.2), and a nitrogen content of 3.15 per cent (calculated = 3.16 per cent). The substance reacts with Type III antipneumococcus serum in dilutions of 1:5,000,000. When weighed, dried samples of this amino compound are dissolved in water, and then 2.2 equivalents of hydrochloric acid added, and the solution titrated at 0°C. with N/20 sodium nitrite, it is found that the derivative uses up exactly the theoretical quantity (calculated on the nitrogen basis) to form the diazonium derivative. Our first few preparations of the amino ether utilized only some 75

per cent of the theoretical quantity of nitrite when titrated. Later preparations, however, appeared to have all of the nitrogen in the amino form, for they utilized the theoretical quantity of nitrous acid.

3. The Coupling of the Aminobenzyl Ether of the Soluble Specific Substance to Serum Globulin

250 mg. of the aminobenzyl ether soluble specific substance were dissolved in 25 cc. of water. The solution was cooled to 0°C. and to it was added 2.2 mols of normal hydrochloric acid. To the opalescent solution was now added 1 mol of N/10 sodium nitrite. The solution was stirred for 20 minutes. Normal sodium hydroxide was then added *very* cautiously until the solution was just neutral to litmus paper. The solution of diazotized soluble substance was now poured into an alkaline solution of serum globulin, (prepared from normal horse serum by repeated precipitation with half saturation of ammonium sulfate) containing 800 mg. of the latter dissolved in 30 cc. of N/2 sodium carbonate at 0°C. An orange color soon developed which deepened on standing, until finally, after 2 hours standing at 0°C., the presence of free diazonium body could no longer be demonstrated. The deeply colored solution was now acidified carefully by the addition of 10 per cent trichloracetic acid. A yellow precipitate separated, which was centrifuged at low speed. The supernatant liquid still contained relatively large quantities of uncombined protein, and some free soluble substance. It was discarded.

The precipitate was suspended in 30 cc. of salt solution and was briskly stirred for 30 minutes to break up any lumps which were present. As soon as a uniform suspension of the yellow precipitate was obtained, it was dissolved by the addition of N/10 sodium hydroxide. The fine particles swelled and gave what was apparently a solution, but a large amount of transparent orange jelly could be centrifuged away from some of the true solution of the substance. However, instead of centrifuging and discarding this jelly, the entire suspension was again acidified with a small amount of trichloracetic acid. The precipitate thus formed was again centrifuged and the clear supernatant liquid was tested for the presence of specifically reacting polysaccharide. Solution and reprecipitation were repeated until no more reactive polysaccharide could be found in the supernatant liquid. This required, in all, about three precipitations.

The final precipitate was now suspended in 30 cc. of 0.9 per cent salt solution containing 0.25 per cent tricresol; N/10 sodium hydroxide was added until the mixture was very faintly alkaline to litmus, but not alkaline to phenolphthalein. Most of the compound went into solution, a small amount still remained in suspension as a jelly-like conglomerate. The mixture was diluted to 150 cc. with 0.25 per cent tricresol salt solution and was used for immunization purposes.

The conjoined carbohydrate-protein reacted with Type III antipneumococcus serum in dilutions of 1:500,000. When the precipi-

tate thus formed was separated by centrifugation, it was found to be colored yellow, and when it was dissolved in alkali, an orange solution was obtained. This was an indication that the specific polysaccharide actually is bound to the protein by way of the chromophoric linkage —N=N— and that when the specific part of the protein-carbohydrate complex reacts with pneumococcus antibody, the complex precipitates *in toto*. The carbohydrate-protein was found to contain 13 per cent of sugar, calculated as glucose. This is in fair agreement with the amount of bound polysaccharide calculated from the reactivity of the carbohydrate derivative with pneumococcus antiserum, *i.e.* roughly 10 per cent, since the sensitivity of this reaction is 1:5,000,000. This polysaccharide-protein complex has been used to immunize animals. The results of the immunological studies are presented in the following paper.

SUMMARY

1. The *p*-amino and *p*-nitromonobenzyl ethers of the specific polysaccharide of Type III Pneumococcus have been prepared.

2. The diazonium ether of the specific polysaccharide has been coupled with serum globulin to yield a specific polysaccharide-protein complex and this complex has been used for immunization. The results of the immunological studies are presented in the following paper.

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