# THE CARBOHYDRATES OF GONOCOCCUS AND MENINGOCOCCUS

## I. The Alcohol-Precipitable Fraction*

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As early as 1874 Scheibler (1) studied a polysaccharide isolated from the gum of *Streptococcus (Leuconostoc) mesenterioides*, an organism of some economic importance to the manufacturers of sugar; but little attention was given to the carbohydrates of pathogenic bacteria until Toenniessen's (2) work on Friedländer's bacillus. Within the past decade and a half, however, advance in this field has been accelerated by the use of immunological methods as an adjunct to the usual techniques of organic chemistry. Zinsser and his coworkers (3, 4) prepared from several organisms residue antigens which were almost protein-free, reacted by precipitation with homologous immune sera, but failed to elicit antibody production in animals. Avery and Heidelberger (5, 6) carried out a most exhaustive and fruitful investigation of the carbohydrates of pneumococcus. They obtained from Types II and III products which were nitrogen-free and chemically distinct from each other and from that of Type I which contained some nitrogen, presumably as an integral part of its molecule. They were non-antigenic in rabbits, but reacted specifically in extremely high dilutions with homologous antisera. From a rough strain of pneumococcus Tillett and Francis (7, 8) obtained a non-type-specific carbohydrate, designated the C fraction. Recently Wadsworth and Brown (9) have reported the isolation of type-specific substances of a carbohydrate nature from each of the fixed types of pneumococci which they regard as different from the polysaccharides of Avery and Heidelberger and also from the C fraction of Tillett and Francis.

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The preparation of carbohydrate fractions from a number of other organisms has been accomplished, but only the work of Casper (10) on gonococcus, and that of Zozaya and Wood (11) and of Webster and Rake (12) on meningococcus relates to the present study.

This study has been carried along parallel with the work on the nucleoproteins reported in the preceding paper (13). It began, therefore, with the preparation and investigation of carbohydrate fractions from gonococcus and meningococcus, the two organisms of special interest to us. Subsequently *Micrococcus catarrhalis*, R pneumococcus, *Streptococcus hemolyticus*, and *Staphylococcus aureus* were added for purposes of comparison.

## *Methods*

The organisms were grown and extracted by the methods described in the preceding paper (13). The supernatant liquid from the acetic acid precipitation of the proteins was filtered, neutralized with sodium hydroxide, and evaporated to small bulk (each liter to 25 cc.) at 56°C., in an air current. This concentrated solution was then made slightly acid and placed in boiling water for 7 minutes to remove the remaining proteins by heat coagulation. The solution was then filtered and added to 7 to 8 volumes of 95 per cent ethyl alcohol. The resulting precipitate was allowed to remain in the alcohol for several hours to facilitate its denaturing action on any traces of protein still present. The supernatant alcoholic solution was decanted, filtered, and evaporated to dryness. The residue from this evaporated alcoholic solution was found to be protein-free by all the protein tests to which it was subjected. Aside from the sodium acetate (from the acetic acid and sodium hydroxide used) it consisted principally of carbohydrate, a fraction which will be the subject of a later report. The alcoholic precipitate contained the fraction which primarily concerns us here. It also proved to be chiefly polysaccharide, but may have included traces of amino acids and any alcohol-insoluble, non-heat-coagulable, non-acid-precipitable proteins. It was centrifuged and drained free from excess liquid. While still damp it was dissolved in water, then twice reprecipitated from 80 per cent alcohol of first slightly alkaline and then slightly acid reaction. Finally the aqueous solution was dialyzed in a cellophane bag against distilled water.

Not all of the preparations were carried through the final step of repeated precipitation and dialysis, because considerable loss in carbohydrate resulted. It should be noted, however, that in immunological behavior such preparations did not differ from those which had been more highly purified.

## *Physical and Chemical Properties*

In the dry state the carbohydrates prepared from all of the organisms were light yellow in color. They were entirely soluble in distilled water

and in 0.9 per cent sodium chloride solution. When ethyl alcohol was added to an aqueous solution precipitation began at an alcoholic concentration of about 40 per cent and increased with each addition of alcohol until the concentration reached 80 per cent. They seemed to be resistant to the action of weak acids and alkalies. They were only partially dialyzable; that is to say, a detectable loss occurred after 10 days dialysis in a bag of cellophane (No. 600) against distilled water. Possibly products of a slow hydrolysis of the polysaccharide were lost (escaped through the membrane).

The tests for protein, *e.g.* biuret and xanthoproteic, were negative, although carbohydrate reactions, as the Molisch test, were strongly positive. The solutions retained in the cellophane bag after dialysis gave negative Benedict-Fehling reduction tests. However, this test was positive when performed on hydrolysates obtained by boiling the carbohydrates with 2 per cent hydrochloric acid.

The preparations most extensively studied were those of gonococcus and meningococcus. Negative ninhydrin reactions indicated the absence of an amino acid impurity in appreciable quantity. The Millon and Hopkins-Cole tests were also negative. Negative orcinol, phloroglucinol, and resorcinol reactions indicated the absence of pentose and ketose radicals. The nitrogen content of the gonococcal polysaccharide was found by Kjeldahl micro determination to be 4.2 per cent. An aqueous solution of this polysaccharide was optically inactive. The nitrogen content of the meningococcal polysaccharide was 3.7 per cent.

*Toxicity for Laboratory Animals.*—Both the gonococcal and meningococcal carbohydrates were non-toxic for rabbits and mice. The former received the material intravenously, the latter intraperitoneally, without evidence of deleterious effect.

*Antigenicity of the Carbohydrate Preparations.*—The sera of rabbits which had been repeatedly injected intravenously with carbohydrate fractions prepared from gonococci and from meningococci contained no antibodies demonstrable by the precipitin reaction. An additional attempt was made, patterned after the method employed so successfully by Landsteiner with his alcohol-soluble haptenes, in which the rabbits were injected intravenously with gonococcal carbohydrate mixed with pig serum. These animals developed high titers of anti-pig serum precipitins but none at all for the carbohydrate.

*Cutaneous Reactions with Gonococcal Carbohydrate.*—In rabbits rendered hypersensitive by the implantation of agar foci containing either gonococci or meningococci (14), allergic reactions of the delayed type were elicited by the intracutaneous injection of 0.1 cc. of a 1:1000 solution of gonococcal carbohydrate. No reaction followed intracutaneous injection into normal (snuffle-free) rabbits.

*Immunological Reactions.*—Precipitin reactions were made in the same fashion and with the same sera as those described in the preceding paper. A summary of representative tests is given in Table I. Cross-reactions occurred between the carbohydrates of the gonococcus and meningococcus and their respective antisera, a result which was to be

TABLE I

*Precipitin Reactions with Carbohydrate Preparations*

| Carbohydrates prepared from | Rabbit sera prepared by immunization with | | | Commercial sera | |
|---|---|---|---|---|---|
| | Gonococcus | Meningococcus | *M. catarrhalis* | Antimeningococcus | Antipneumococcus Type III |
| Gonococcus | +++ ++ | +++ | − | +++ +++ | +++ +++ |
| Meningococcus | +++ ++ | +++ ++ | − | +++ +++ | +++ ++ |
| *M. catarrhalis* | +++ + | − | +++ + | +++ + | +++ |
| R pneumococcus | +++ + | − | − | +++ + | +++ + |
| *Strep. hemolyticus* | − | − | − | − | +++ |
| *Staph. aureus* | − | − | − | − | − |

The plus marks indicate dilutions of precipitinogen as multiples of 10; thus: +++ = 1:1000; +++ + = 1:10,000; etc. − = negative in dilution of 1:1000.

expected. The carbohydrate of *M. catarrhalis* was precipitated by antigonococcal serum, while the anti-*catarrhalis* serum failed to react with the polysaccharides of either of those organisms. An inconsistency is to be noted in the case of *M. catarrhalis* which reacted with commercial antimeningococcal serum but not with that prepared in the laboratory by the immunization of rabbits. This may be explained by the fact that the titer of the rabbit serum was lower (only 1:100,000 for the carbohydrate of the homologous organism). The immunizations of the rabbits were purposely carried only to the point at which they yielded sera of workable titers in the hope that their specificity might be sharper.

Sera prepared by the immunization of rabbits with the "nucleoproteins" of gonococcus and meningococcus gave the same reactions as the antibacterial sera, though usually in somewhat lower titer.

Antipneumococcus Serum Type III reacted with the carbohydrates of gonococcus and meningococcus in dilutions of 1:1,000,000 and 1:100,000 respectively, and with the carbohydrates of *M. catarrhalis* and hemolytic streptococcus in dilutions of 1:1000. Carbohydrates isolated from an R pneumococcus (one prepared by the method herein described and one by the method of Tillett and Francis (7)) reacted in dilutions of 1:10,000 not only with antipneumococcus serum of all three types but with antimeningococcus and antigonococcus serum as well.

*Strain Specificity.*—Carbohydrate fractions prepared from 6 strains of gonococcus employed in precipitin reactions with immune sera to 5 strains of that organism showed no evidence of strain specificity.

*Reactions with Other Sera.*—Gonococcal and meningococcal polysaccharides gave negative precipitin reactions with commercial sera to the following organisms: typhoid, paratyphoid A and B, dysentery and anthrax bacilli, and several varieties of streptococci. Negative results were also obtained with diphtheria and scarlet fever antitoxins.

## DISCUSSION

Since the classical investigations of Landsteiner, evidence has accumulated to support his original conception of the nature of antigenic specificity. It is now generally accepted that the specificity of pure proteins may reside in individual radicals, while that of highly complex antigens may depend upon components of relatively simple chemical composition. These components in the case of a number of bacterial antigens have been found to be polysaccharides, and the carbohydrates herein described are also so regarded. No evidence was obtained to preclude the assumption that they exist *in vivo* as part of a glycoprotein. As noted in the preceding paper, the "nucleoproteins" described gave strongly positive Molisch reactions. Furthermore sera prepared by immunization with gonococcal and meningococcal "nucleoproteins" reacted with the protein-free carbohydrates of those organisms.

Webster and Rake (12) in a preliminary communication report the

isolation from meningococcus of a type-specific as well as a non-specific polysaccharide. Until the publication of their methods we cannot compare our work with theirs, but the conjecture seems obvious that the carbohydrates herein described correspond to their non-specific polysaccharide.[1] It should be noted that our preparations were made from thrice washed organisms, and it is quite possible that a more specific constituent was lost in this process.

As it has been impossible to demonstrate capsules on gonococci or meningococci, the carbohydrates described have been regarded as somatic in origin, and therefore analogous to the C fraction isolated by Tillett and Francis (7) from rough pneumococcus.

The cross-reactions between the carbohydrates of gonococcus and meningococcus and antisera to those two organisms were to be expected from their biological similarity. So were the reactions of *catarrhalis* carbohydrate, which were positive with antigonococcus and negative with antimeningococcus serum, and likewise the failure of anti-*catarrhalis* serum to react with the carbohydrates of the other two *Neisseriae*. For these findings are in harmony with the relationship suggested by the work of Miller and Castles (14) on the allergic cutaneous reaction to these organisms.

The precipitin reactions of Antipneumococcus Serum Type III with the carbohydrates of gonococcus and meningococcus, however, were quite unexpected. They are doubtless analogous to the several other hetero-antigenic relationships among bacteria which have been reported; *e.g.*, between Strain E of Friedländer's bacillus and Type II pneumococcus reported by Avery, Heidelberger, and Goebel (15), and meningococcus, *B. anthracis*, *B. subtilis*, *B. proteus*, and *B. mesentericus* by Zozaya (16). The latter subsequently (17) qualified his statements about these cross-reactions, in the light of his observation that bacteria grown on solid media may adsorb enough agar to engender antibodies to it. We, ourselves, had considered this possibility (suggested by Furth and Landsteiner (18)), as well as the possibility that traces of undigested egg white which had failed to precipitate in the preparation of our media might adhere to the bodies of the organisms and cause

[1] Since the presentation of this manuscript for publication, the study referred to has been reported under the authorship of Rake and Scherp (20).

the hetero-reactions which we encountered. Control tests were accordingly run with the agar medium as precipitinogen; but they were all negative and therefore received no mention in our preliminary communication (19). As an additional check on the egg white digest medium as a source of non-specific reactions, rabbits were immunized to egg white, but their sera failed to react with our carbohydrate and nucleoprotein preparations, although they contained anti-egg white precipitins in very high titers. Since the publication of Zozaya's paper (17) these control tests have been repeated. With the following exceptions the results duplicated those obtained 2 years ago. These exceptions warrant description. Agar, in dilution of 1:1000, when superimposed on certain of the commercial antimeningococcus and antipneumococcus sera,[2] showed at the end of an hour the formation of a translucent flocculus above the interface of the two liquids. It was much greater in bulk than the heaviest of precipitin rings, but was unlike one in consistency, and showed no tendency to settle, even to the upper surface of the serum. The highest dilution of agar which produced this reaction was 1:10,000 with antimeningococcus, and 1:1000 with certain antipneumococcus sera, irrespective of type. Whether or not this phenomenon should be regarded as a precipitin reaction is being left for the present an open question.

More pertinent to the problem at hand are the negative control tests with our rabbit immune sera (on egg white, media, and agar), and the fact that carbohydrates prepared from gonococci and meningococci grown in liquid media gave the same non-specific cross-reactions as those prepared from organisms grown on agar medium. It seems highly improbable, therefore, that the cross-reactions herein described are due to agar adsorbed by the bacteria during their cultivation.

As has been noted, the carbohydrates described above are precipitable by alcohol from bacterial extracts after the removal of their "nucleoproteins." In the case of gonococcus and meningococcus two other fractions are at present under investigation: an alcohol-soluble carbohydrate and a bacterial residue of difficult solubility which seems also to be composed of, or to be rich in carbohydrate.

[2] These sera were from different lots, as those used 2 years ago were no longer available.

SUMMARY

The alcohol-insoluble polysaccharides of gonococcus and meningococcus were found to contain 4.2 and 3.7 per cent nitrogen respectively, to be protein-free by chemical test, to reduce Fehling-Benedict solution only after hydrolysis. They were non-toxic for rabbits and mice, and failed to engender antibodies (precipitins) in rabbits. They produced no cutaneous reactions in normal, snuffle-free rabbits, but caused typical allergic reactions of the delayed type in rabbits rendered hypersensitive to these organisms. Both carbohydrates reacted in high dilution with Antipneumococcus Serum Type III. For comparison, carbohydrates were prepared also from *Micrococcus catarrhalis*, *Streptococcus hemolyticus*, *Staphylococcus aureus*, and a rough strain of pneumococcus.

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