A STUDY ON BACTERIAL PROTEINS WITH SPECIAL CONSIDERATION OF GONOCOCCUS AND MENINGOCOCCUS*

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The isolation of "nucleoproteins" from microorganisms dates back to Stutzer's (1) chemical investigation of yeast in 1882; but their first identification in one of the common pathogens seems to have been the result of Aronson's (2) work on the diphtheria bacillus in 1900. Since that time a considerable literature has accumulated. When the mere presence of "nucleoproteins" in bacteria ceased to be a novelty, and their analysis proved too difficult for the classical techniques of biochemistry, interest shifted to their immunological behavior.

The few references which follow are to studies particularly pertinent to the problem in hand. Heidelberger and Avery (3, 4) prepared from pneumococci a "purified nucleoprotein" which was capable of engendering antibodies, and in its antigenic behavior exhibited species specificity but not type specificity. Lancefield (5) in 1925 studied the "nucleoproteins" of several strains of *Streptococcus viridans*, and subsequently (6) those obtained from hemolytic streptococci. Heidelberger and Kendall (7) fractionated the protein of a scarlatinal streptococcus. An exhaustive review of the published work on the tubercle bacillus is to be found in the recent book by Wells and Long (8).

The work reported below began as a study of the chemical and immunological properties of the protein and non-protein fractions obtained from gonococcus and, for purposes of comparison, from meningococcus. The unexpected finding of positive cross-reactions with immune serum to an unrelated species (Pneumococcus Type III) as reported in our preliminary communication (9) led to an extension of the investigation to include several other organisms. "Nucleoproteins" were therefore prepared from *Micrococcus catarrhalis*, *Staphylococcus aureus*, *Streptococcus hemolyticus*, and an R strain of pneumococcus.

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Methods

The organisms were cultivated in ordinary 16 ounce medicine bottles on an agar medium[1] consisting of a tryptic digest of egg white (from which the heat-coagulable proteins had been removed), dextrose, and an appropriate mixture of salts. The 12 to 18 hour growth was removed with 0.9 per cent sodium chloride solution, filtered through a thin layer of cotton supported by gauze, separated by centrifugation, and washed thrice by alternate suspension in normal salt solution and centrifugation.

Organisms grown in liquid media were also employed in the preparation of bacterial nucleoprotein. In the case of gonococcus and meningococcus this medium contained the same ingredients as the solid, less the agar (11). For pneumococcus ordinary meat infusion broth was used. After incubation for 18 hours the organisms were recovered by centrifugation and treated in the same way as those grown on solid media. No differences were detected in the immunological behavior of the nucleoproteins prepared from a given organism grown on solid or in liquid medium. The former therefore became the routine method as it was the more efficient.

Extraction of the so called "nucleoprotein" from the bodies of the organisms was accomplished by the several methods described below. The procedures varied from rather drastic chemical treatment to simple mechanical disintegration of the cellular structure. The method employed at the beginning of this study was that which Lancefield had found satisfactory for the preparation of her streptococcal nucleoproteins, briefly, extraction with N/100 sodium hydroxide. It was soon found, however, that alkali in considerably lower concentrations would extract the proteins of gonococcus and meningococcus, and the method was accordingly modified to the one described under Section 6 below. But when certain unexpected cross-reactions were encountered, consideration was given to the possibility that alkali even so dilute might have denatured the proteins. For purposes of control, therefore, preparations were made by such innocuous, though relatively inefficient methods as to rule out this possibility.

Stained smears of the organisms were examined microscopically to compare the effects of the various treatments on their morphology. Only insignificant changes were observed in the contour of the Gram-negative cocci, though the cellular content of stainable material was reduced roughly in proportion to the quantity of alkali to which they had been exposed; *i.e.*, to their loss of protein by extraction.

1. Extraction with Distilled Water.—The well packed bacteria were suspended in 3 to 4 volumes of cold distilled water and allowed to stand for 12 hours at 4–8°C. The yield from this extraction was very small, as was to be expected, since the true nucleoproteins and many other protoplasmic constituents are insoluble in water.

2. Maceration with Sodium Chloride.—To 20 cc. of fresh, moist organisms, well packed by centrifugation, were added 22.5 gm. of fine crystals of sodium chloride,

[1] Described as the control medium in the paper by Miller, Hastings, and Castles (10).

and the mixture was vigorously ground in a mortar from time to time during 1 day. After standing overnight at room temperature, 2.5 liters of distilled water were added, a quantity sufficient to reduce the concentration of sodium chloride to about 0.9 per cent. It was presumed that the cells might take up sufficient salt during their maceration to cause their disruption when the distilled water was added. This method was somewhat more efficient than the first. The yield was further increased by the addition of sufficient solid sodium carbonate to the salt crystals to make a final concentration of 0.27–0.5 per cent.

3. Grinding in the Dry State.—Fresh organisms were spread in a thin layer on a watch-glass and dried *in vacuo* over sulfuric acid. They were then ground for 72 hours in a rotary ball mill and subsequently extracted with distilled water.

4. Freezing and Thawing.—Moist, packed organisms were rapidly frozen and thawed 4 times, then suspended in water. Microscopical examination showed most of the cells to be still intact.

5. Surface Tension Depressants.—Attempts were made to effect lysis of the organisms by purified solutions of sodium oleate, sodium ricinoleate, and sodium taurocholate. With the exception of pneumococcus, which was promptly dissolved by each of these, the result was no better than that accomplished by simple alkalinization to the same degree. (See Section 6 below.) Difficulties were encountered in preparing the proteins from these extracts as traces of the soaps could not be removed and gave false precipitin reactions.

6. Treatment with Dilute Alkali.—Organisms were suspended in 10–15 volumes of cold distilled water containing a trace of phenol red as indicator. Sufficient sodium hydroxide was added to bring the reaction to pH 7.6–7.8. The suspension was kept in the refrigerator at 4–8°C. for 12–18 hours, during which time the reaction was maintained at pH 7.6–7.8 by the addition, whenever necessary, of sodium hydroxide. It was found that a batch of 20 cc. of packed organisms, suspended in 2.5 liters of water, usually required a total of about 25 cc. of N/100 sodium hydroxide. This method was found to be the most satisfactory and was employed in preparing the proteins from the three Gram-negatives and pneumococci used in the experiments which follow. It was chosen because it was more efficient than the foregoing and yielded a product indistinguishable immunologically from those obtained by simpler means. In the case of the staphylococcus and streptococcus, satisfactory extraction was accomplished only in N/100 NaOH, so that method was necessary in the preparation of their proteins.

The products obtained by these various methods of extraction are doubtless of different composition, since the components of so called "bacterial nucleoprotein" (albumin, globulin, mucin, nucleoprotein, etc.) differ in their respective solubilities in water, dilute alkali, and normal saline; but the variation in composition was regarded as quantitative rather than qualitative because all of the preparations from a given organism gave the same immunological reactions.

Preparation of the Protein.—The extract was freed by centrifugation from insoluble matter in suspension, and to it was slowly added, with constant stirring, dilute acetic acid, in the least quantity required to give a maximum precipitate.

After the heavy precipitate had settled, the supernatant liquid was decanted and used in the preparation of the carbohydrate described in the following paper (12).

The precipitate was dissolved in sodium hydroxide solution, at pH of about 7.8, reprecipitated with acid, and centrifuged. This precipitation was repeated once more. Then the protein was dissolved and dialyzed in bags of cellophane (No. 600) against distilled water for several days. During dialysis the protein solution was covered by a protecting layer of toluene. The dialyzed solution was evaporated to dryness in an air current at 56°C. and preserved in the dry state.

A later paper will report our findings on the peculiar nature and immunological effect of some of the individual proteins obtained by fractionating these protein mixtures.

Properties

The bacterial protein used in these experiments is designated "nucleoprotein" (in accordance with common usage), although it is admittedly a mixture of acid-precipitable cellular proteins, including albumins, globulins, etc., as well as the true nucleoproteins. All of the preparations were partially soluble in distilled water and entirely soluble in a solution made alkaline, pH 7.6, with sodium hydroxide. They gave all the usual protein reactions; *e.g.*, biuret, xanthoproteic, Millon, Hopkins-Cole, sulfosalicylic acid and phosphotungstic acid tests. A positive Molisch test on each of them indicated the presence of a carbohydrate radical.

Toxicity for Laboratory Animals.—Intravenous injection of saline solution of gonococcal or meningococcal nucleoprotein in any considerable amounts proved lethal to rabbits. A series of experiments was therefore performed to compare the relative toxicity of these two organisms with their respective nucleoproteins. In the interest of economy of material, 20 gm. mice were used as the test animal and intraperitoneal injection as the method of administration.

Freshly grown organisms were spread on a watch-crystal, desiccated *in vacuo*, and taken up in sufficient saline to make a 0.5 per cent suspension on a basis of dry weight. In the case of meningococcus the suspension was heated at 56°C. for an hour. The nucleoproteins were likewise used in 0.5 per cent solutions. The concentration of protein in the bacterial suspensions was therefore somewhat less than in the nucleoprotein solutions, but, since no quantitative determinations were made of the organisms' actual content of this constituent, more accurate equivalence was impossible. The results were found to be significant, this small discrepancy notwithstanding.

Several experiments indicated the minimum lethal dose of gonococci for white mice to be between 2.5 and 5.0 mg. and that of the nucleoprotein to be only slightly greater. As the error inherent in such a method of biological assay can be corrected only by the use of numbers of animals great enough to be treated statistically, these figures must be regarded as close approximations. The experiment in Table I shows, however, that mice succumbed more quickly to injections of whole organisms than to equal quantities of nucleoprotein of the same strain.

TABLE I

Relative Toxicity for Mice of Gonococci (Intact Organisms) and of Nucleoprotein Prepared from the Same Strain

Material and dose		No. of mice injected	No. of mice surviving at the end of				Total dead
			12 hrs.	18 hrs.	36 hrs.	60 hrs.	
	mg.						
Organisms.....	1.25	3	3	2	2	2	1
Nucleoprotein.....	1.25	3	3	3	3	3	0
Organisms.....	2.5	5	3	3	1	0	5
Nucleoprotein.....	2.5	5	5	5	4	4	1
Organisms.....	5.0	5	3	0	0	0	5
Nucleoprotein.....	5.0	5	5	4	0	0	5
Organisms.....	10.0	10	7	1	0	0	10
Nucleoprotein.....	10.0	10	9	3	2	2	8

Table II gives the results of a similar experiment designed to compare the toxicity of meningococcus with that of nucleoprotein prepared from the same strain. It indicates that intact meningococci were somewhat more toxic than their nucleoprotein, the difference here being more apparent than in the case of gonococcus. That the heating of the bacterial suspension did not appreciably reduce its toxicity can be confidently assumed, for this property has been found to be remarkably resistant to heat. A number of experiments (13) have, in fact, shown that prolonged exposure to much higher temperatures is necessary to diminish perceptibly the lethal action of meningococci for mice. In this connection should be noted the work of Branham and Lillie (14)

on experimental meningitis in guinea pigs. They found that intracisternal inoculations of boiled and living meningococci produced essentially identical clinical and histopathological pictures.

Effect of Defatting.—Extraction with acetone and ether in the cold did not appreciably alter the toxicity for mice, nor the antigenic properties, as measured by precipitin reactions, of gonococci and meningococci (whole organisms) and their nucleoproteins.

Allergic Reactions with Nucleoproteins.—Rabbits rendered hypersensitive to gonococci and meningococci by the subcutaneous implanta-

TABLE II

Relative Toxicity for Mice of Meningococci and of Nucleoprotein Prepared from the Same Strain

Material and dose		No. of mice injected	No. of mice surviving at the end of				Total dead
			12 hrs.	18 hrs.	36 hrs.	60 hrs.	
	mg.						
Organisms	0.62	5	4	4	4	4	1
Organisms	1.25	5	3	3	2	2	3
Nucleoprotein	1.25	5	5	5	5	5	0
Organisms	2.5	5	3	3	2	1	4
Nucleoprotein	2.5	5	3	3	2	2	3
Organisms	5.0	5	4	2	0	0	5
Nucleoprotein	5.0	5	4	3	3	2	3
Organisms	10.0	10	1	0	0	0	10
Nucleoprotein	10.0	10	7	4	3	2	8

tion of masses of infected agar (see Miller and Castles (15)) were found to be equally allergic to their nucleoproteins. The reactions were always of the delayed type. When gonococcal nucleoprotein was used as the sensitizing ingredient in the subcutaneous agar foci, the rabbits developed an even higher degree of allergy than to viable gonococci. The nature of the inflammatory reaction about these foci was not studied. No difference was detected in the specificity of the hypersensitiveness evolved by nucleoprotein and by the organisms themselves.

Immunological Reactions

Preparation of Immune Sera.—Antisera were prepared from the nucleoproteins of gonococcus, meningococcus, and *M. catarrhalis.* Snuffle-free rabbits weighing from 4–6 pounds were used. They received by intravenous injections at 4 to 5 day intervals, a 1 per cent solution of the protein in saline adjusted to pH 7.6–7.8. The doses were increased progressively (1, 2, 4, 6, 8, and 10 cc.), and the animals were bled 5 days after the last injection. It should be noted that this slight alkalinization seemed to diminish somewhat the toxicity of the protein.

Antibacterial sera were also prepared, in the case of gonococcus, meningococcus, *M. catarrhalis,* and an R pneumococcus, by intravenous immunization of rabbits with intact organisms suspended in saline. These immunizations were effected more slowly as the greater toxicity of the inocula demanded greater caution.

Precipitin Reactions.—The precipitin tests were made by the contact method in which serum diluted 1:3 with 0.9 per cent sodium chloride solution was placed under progressive 10-fold dilutions of the protein in normal saline, usually beginning at 1:1000. The titers of the antisera were designated as the highest dilution of antigen which showed definite clouding at or near the intersurface of the two liquids when examined in bright illumination against a black background. This method eliminates the variability of precipitation commonly known as inhibition zones. The tests were made in small vials (26 x 5 mm.) which had been thoroughly cleansed with hot chromic acid cleaning solution and many applications of distilled water.

Results.—The results of a series of precipitin tests on nucleoprotein preparations from five strains of gonococcus with five representative immune sera are given in Table III. Two of the sera were obtained by immunization with whole organisms (individual strains) and two with the nucleoproteins of those same strains, while the fifth was prepared by immunization with a mixture of several strains, including the foregoing. It will be noted that the titers vary from 1:10,000 to 1:1,000,000, but that the sera did not react to their homologous proteins in unusually high dilutions. Nor did the polyvalent serum show differences from the rest. The titers of the antiprotein sera averaged about the same as the antibacterial.

Table IV presents the results of precipitin reactions in which the nucleoproteins of 6 organisms served as antigens, and the following as precipitating sera: antigonococcus, antimeningococcus, and anti-*catarrhalis* sera obtained by the immunization of rabbits, and the three antipneumococcus sera commonly used for typing pneumococci. Gonococcal nucleoprotein was precipitated by antimeningococcus

serum and by antipneumococcus sera of all three types, but not by anti-*catarrhalis* serum. The latter, nevertheless, gave a positive reaction with meningococcal nucleoprotein, which was precipitated by only Type III of the antipneumococcus sera. Positive reactions were

TABLE III

Precipitin Reactions with Gonococcal Nucleoproteins Prepared from Different Strains

Nucleoprotein strain	Sera prepared by immunizing rabbits to									
	Strain 1				Strain M_6B_2				Organisms of several strains	
	Nucleoprotein		Organisms		Nucleoprotein		Organisms			
1	+++	+	+++	++	+++	+++	+++	+++	+++	+++
3	+++	+	+++	+	+++	++	+++	++	+++	+
5	+++	++	+++	++	+++	++	+++	++	+++	++
M_6B_2	+++	+	+++	++	+++	++	+++	++	+++	++
10	+++	++	+++	+++	+++	++	+++	+++	+++	++

In this and the following tables, the plus marks indicate dilutions of precipitinogen as multiples of 10; thus: +++ = 1:1000; +++ + = 1:10,000, etc. − = negative in dilution of 1:1000.

TABLE IV

Precipitin Tests with Nucleoproteins Showing Non-Specific Cross-Reactions

Nucleoproteins prepared from	Sera								
	Anti-gonococcus		Anti-meningococcus		Anti-*catarrhalis*		Antipneumococcus		
							Type I	Type II	Type III	
Gonococcus	+++	++	+++		−		+++	+++	+++	+
Meningococcus	+++	++	+++	+++	+++	+	−	−	+++	+
M. catarrhalis	+++		+++		+++	+	−	−	+++	
R pneumococcus	+++	+	−		−		+++	+++	+++	+
Strep. hemolyticus	−		−		−		−	−	+++	
Staph. aureus	−		−		−		−	−	+++	

obtained with the nucleoprotein prepared from an R strain of pneumococcus and antigonococcus, but not with antimeningococcus nor anti-*catarrhalis* sera.

Effect of Tryptic Digestion of the Nucleoprotein.—Gonococcal and meningococcal nucleoproteins were subjected to digestion by trypsin

and then tested for certain of the cross-reactions, together with nucleoprotein solutions which had been alkalinized (to pH 7.8) and heated (to 56°C.) the same as the digests. The results presented in Table V show that both nucleoproteins failed after digestion to react with Antipneumococcus Serum Type III, but continued to react with sera to closely related organisms (the other *Neisseriae*).

Precipitin Reactions with Other Sera.—Both gonococcal and meningococcal nucleoproteins failed to react when tested with a variety of

TABLE V

Precipitin Reactions with Tryptic Digests of Gonococcal and Meningococcal Nucleoproteins

Precipitinogen	Immune sera			
	Anti-gonococcus	Anti-meningococcus	Anti-*catarrhalis*	Antipneumococcus Type III
Gonococcal nucleoprotein	+++ +	+++	Not made	+++ +
Gonococcal nucleoprotein after tryptic digestion	+++ +	+++		−
Meningococcal nucleoprotein	+++	+++ ++	+++ +	+++ +
Meningococcal nucleoprotein after tryptic digestion	+++	+++ ++	+++ +	−

other sera, which included typhoid, paratyphoid A and B, dysentery, anthrax, and human serum containing a high titer of agglutinins for *B. melitensis*.

DISCUSSION

As has been mentioned, the term "nucleoprotein" is employed in accordance with the usage of bacteriologists rather than of chemists, for it designates not a chemical entity but a mixture of substances including the true nucleoproteins. The present report concerns certain properties of the nucleoproteins of gonococcus and meningococcus, and for purposes of comparison, of *M. catarrhalis*, R pneumococcus, *Streptococcus hemolyticus*, and *Staphylococcus aureus*.

Both gonococcal and meningococcal nucleoproteins engendered precipitins in rabbits which were indistinguishable by cross-reactions with

other proteins from the precipitins engendered by the intact organisms of these two species. In other words, the antigenic factors responsible for these cross-reactions were neither destroyed nor created by the chemical treatment involved in their preparation, which, for most of our material, included dilute alkali. In the case of gonococcus, the harmlessness of our standard method was also checked by making several small lots which were extracted by simple physical means without the use of alkali. These preparations, with the exception of those subjected to the action of soaps of fatty acids, showed no differences from the others which could be detected by the immunological technique employed.

The cross-reactions between gonococcal nucleoprotein and antipneumococcus serum of all three types, as well as those between meningococcal and *catarrhalis* nucleoproteins and Type III serum, are, therefore, regarded as heterogenetic reactions. It is interesting that the antigenic factor responsible for this reaction was destroyed by tryptic digestion, although those responsible for the reactions with their homologous sera and for cross-reactions with those to closely related species (the other Gram-negative diplococci) were not disturbed by such treatment.

In the phenomenon of bacterial allergy nucleoproteins were found to play the same rôle as viable organisms; for the nucleoproteins of gonococci and meningococci evoked, in hypersensitive rabbits, quite as strongly positive cutaneous reactions as did the organisms themselves. And in its ability to induce the allergic state, gonococcal nucleoprotein (the only one so studied) seemed to be even more effective than living organisms. This observation may be explained by its solubility in the body fluids, hence its easier availability to the tissues of the animal. The specificity of the hypersensitiveness so induced and of the cutaneous reactions to nucleoprotein was no sharper than to intact organisms.

The toxicity of both gonococcal and meningococcal nucleoproteins, as determined by their lethal action in mice, was found to be only slightly less than that of the intact organisms of those two species. This indicates that most, if not all, of the toxic property of these organisms is due to some constituent of the fraction designated "nucleoprotein," which is regarded as existing naturally within the

body of the bacterial cell. We have been unable, in fact, to demonstrate any "toxin" in cultures of these organisms either in liquid or on solid media until they had reached an age when some members of the population had begun to disintegrate, or unless the hydrogen ion concentration in the surrounding liquid was such as to favor the extraction of nucleoprotein from the cells.

Investigation of the toxicity as well as the immunological behavior of the products of further fractionation of these proteins is now in progress.

SUMMARY AND CONCLUSIONS

Methods of preparation and certain properties of the "nucleoproteins" of the following organisms are described: gonococcus, meningococcus, *Micrococcus catarrhalis*, R pneumococcus, *Streptococcus hemolyticus*, *Staphylococcus aureus*. No essential differences between the nucleoproteins and the intact cells of gonococcus and meningococcus were observed in their ability to engender immune substances (precipitins), to induce bacterial allergy in rabbits, or to elicit cutaneous reactions (of the delayed type) in rabbits rendered hypersensitive to these organisms. Measured by their lethal action in mice, the toxicity of gonococcal and meningococcal nucleoproteins was found to be but slightly less than that of the intact cells. It seems probable, therefore, that the toxic action of these organisms is due, chiefly or entirely, to some constituent of the nucleoprotein fraction. Extraction with acetone and ether in the cold did not reduce appreciably the toxicity of these organisms and their nucleoproteins, nor alter their immunological behavior.

Cross-precipitin reactions suggested that gonococcal nucleoprotein contains an antigenic factor in common with the non-encapsulated pneumococcus cell, and meningococcal nucleoprotein one in common with the capsular material of Pneumococcus Type III. Tryptic digestion destroys these antigenic factors, but not those responsible for the cross-reactions within the genus *Neisseria*.

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