STUDIES IN THE RELATION OF THE HEMOLYTIC STREPTOCOCCUS TO RHEUMATIC FEVER

II. Fractionation of the Hemolytic Streptococcus by High Speed Centrifugation*

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The literature on serologic investigations of rheumatic fever by streptococcal substances has been reviewed elsewhere (1). It was noted there that the sera of patients with acute rheumatic fever react similarly to those of patients convalescing from streptococcal infections with respect to two streptococcal antigens, the hemolysin and the fibrinolysin. Although these data have added serologic support to the epidemiologic evidence for a relation between the hemolytic streptococcus and rheumatic fever, they have failed to identify either of these streptococcal products as possible agents in the pathogenesis of rheumatic fever. It was pointed out, however, that the failure of such indentification in the case of two streptococcal antigens, out of the considerable number of such antigens which exist, leaves a wide area of possible immunologic relation. Accordingly, the present studies involve a systematic exploration of the immunologically distinct constituents and products of the hemolytic streptococcus, in an attempt either to identify or to rule out a streptococcal agent or mechanism as playing a part in rheumatic fever.

The first step in the immunologic phase of this work has been a reexamination of the antigenic structure of the hemolytic streptococcus. Of its constituent antigens, those most clearly defined and best studied have been the ones concerned in serologic classification: the type-specific M protein by Lancefield (2, 3) and by Zittle (4), the group-specific carbohydrate by Lancefield (5) and by Zittle and Harris (6), and the type-specific T substance by Lancefield and her collaborators (7). The remaining constituents, which comprise the bulk of this organism, have, however, been less clearly defined. There have been three major attacks on this problem, that of Lancefield, of Heidelberger *et al.*, and of Mudd and coworkers. Lancefield (3, 8) found a broadly specific nucleoprotein, "P," in the substance of the streptococcal cell and suspected, but was unable to prove, the presence of another protein (3, 9). Heidelberger *et al.* (10–13) obtained chemical fractions lettered from D to K. Of these, the E to K fractions

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all showed serologic cross-reaction, and absorbed antibodies to all the fractions from antistreptococcal sera. The D fraction did not, however, absorb out antibodies to the E and K fractions. All of these contained nucleic acid. Mudd and Lackman (14) found a single nucleoprotein in the sonic extract of the hemolytic streptococcus, which was shown by Mudd and Wiener (15) to have broad specificity. It was shown by Zittle (4) that this fraction gave tests for both ribose and desoxyribose nucleic acids. Sevag *et al.* (16) isolated heavy particles from sonic extracts of hemolytic streptococcus by high speed centrifugation. They found that the particles were antigenic in rabbits and appeared to contain the group-specific carbohydrate.

The first step in our investigations was to reexamine the constitution of the hemolytic streptococcus so that each distinct antigen comprising the organism could be studied with respect to the immunologic reactions of the rheumatic subjects. The means adopted was that of physical separation of the constituents of the organism rather than chemical fractionation.

Methods and Materials

The streptococci used in these studies included the following strains of group A: NY 5, a strain widely used for the production of erythrogenic toxin; C 203, a type 1 strain also isolated from a patient with scarlet fever; 1048 M, a strain which maintains its production of the type-specific antigen in the laboratory; and H 44, a hyaluronidase-producing strain.[1] The organisms were cultivated in dextrose broth or tryptose phosphate broth with added sodium bicarbonate and phenol red. Some cultures were grown in protein-free medium which was essentially a dialysate of Difco dextrose broth. In addition, large amounts of streptococcal cell sediment (strain NY 5) were made available through the courtesy of the Lederle Laboratories and of Parke, Davis and Company.

The cultures of streptococci were incubated overnight at 37°C. and then neutralized with 4 N sodium hydroxide and reincubated. This was continued until there was no evidence of increased growth. The organisms were then collected by centrifugation, washed twice in physiologic saline solution, and suspended in distilled water at a final concentration usually corresponding to 0.03 gm. of dry weight per cc. This suspension was subjected to high frequency sonic vibration in a magneto-striction oscillator[2] (17) in order to disintegrate the organisms. The milky suspension which resulted was further diluted with one volume of distilled water, aliquots were removed for determination of the total concentration of the suspension in terms of dry weight per cc., and the material was subjected to differential centrifugation. The high speed centrifugation was done in a motor driven centrifuge run in an evacuated chamber (18).

Complement fixation tests were done by incubating the dilution of serum in a volume of 0.4 cc. with 0.1 cc. of the dilution of antigen and 0.1 cc. of complement. The dilution of complement was such that 0.1 cc. contained 1.3 to 1.5 units. under the conditions of the test. After incubation of the mixture at 37°C. for 45 minutes, 0.1 cc. of rabbit anti-sheep–erythrocyte serum, diluted 1:1000, and 0.1 cc. of a 4 per cent suspension of sheep erythrocytes were

[1] The first three of these strains were from the collection of the Department of Bacteriology, School of Medicine, University of Pennsylvania. For the subculture of the H 44 strain the author is indebted to Dr. Karl Meyer.

[2] Manufactured by the Raytheon Manufacturing Company, Boston.

added to each tube. The tubes were incubated at 37°C. for 30 minutes, and the degrees of hemolysis were read as 0, trace, weak, strong, and complete.

Analyses for total nitrogen were done by the micro-Kjeldahl technic, using a $CuSO\text{-}K_2SO_4\text{-}H_2SO_4$ digestion mixture, with superoxol to complete the digestion. The indicator was a mixture of methyl red and methylene blue. Seventieth normal hydrochloric acid was used for the titration, and a steel-gray color was taken as the end-point. The analyses for total phosphorus were done by the method of King (19). It was found necessary, however, to add the molybdate reagent and the sulfonic acid in succession and then to read the color immediately in the photoelectric colorimeter in order to avoid non-specific increments of color. Quantitative estimations of desoxyribose nycleic acid were performed by the method of Dische (20) and measurement of ribonucleic acid by that of Mejbaum (21).

RESULTS

The Fractions.—As a result of experimentation with speeds and lengths of time of centrifugation of suspensions of disintegrated streptococci it was found that an insoluble residue (R) was brought down by centrifugation at 5000 R.P.M. for 30 minutes. The very few organisms which had not been disintegrated were completely removed in this step. The supernate of this centrifugation was a highly opalescent fluid. If this was subjected to repeated centrifugation for periods of 1 hour, first at 15,000 R.P.M. and then at 30,000 R.P.M., a series of opalescent yellowish pellets was obtained, which could be resuspended into a white opalescent suspension. The pellets decreased markedly in size after the 3rd hour at 30,000 R.P.M. (90,000 G) but minute pellets continued to appear even after 9 successive 1 hour centrifugations. Particles of the same range of size as those comprising these pellets had been identified in the cytoplasm of cells of various organs, primarily by Claude (22). In conformity with his nomenclature they were named cytoplasmic particles (CP). The supernate from 8 hours of centrifugation at 30,000 R.P.M. was found to contain a considerable amount of protein. This fraction, after dialysis to remove small molecules, was designated the supernate (S). Since there was both electrophoretic and immunologic evidence that the later pellets obtained at 30,000 R.P.M. were mixtures of the CP and S, only the first two sets of pellets derived at that speed were included with the pellets of the centrifugation at 15,000 R.P.M. in the preparation of the cytoplasmic particles.

It was found that complete separation of the CP and S by centrifugation was impossible. In the case of the CP, any repeated centrifugation in an attempt to purify the material resulted in loss of stability in suspension. On the other hand the smallest units with the immunologic and chemical characteristics of CP could be brought down only by centrifugal fields which sedimented some of the S fraction. In the work to be reported here each of the fractions was not entirely free of the other.

Serologic Reactions of the Fractions

Both soluble fractions reacted with antistreptococcal sera in precipitation and complement fixation tests. The differences between the serologic character of

the CP and S fractions are better shown by their characteristics in complement fixation tests, of which a typical example is shown in Table I.

Complement Fixation.—Since nothing was known of the serologic reactions of these fractions, each was tested against antistreptococcal sera by complement fixation tests in which both antiserum and antigen were progressively diluted.

TABLE I

Complement Fixation of the Streptococcal Nucleoproteins with Rheumatic and Antistreptococcal Sera

	Serum R 26 (rabbit antistreptococcal)									Serum 1268 (active rheumatic)								
	Dilution 1:									Dilution 1:								
	8	16	32	64	128	256	512	1024	2048	4	8	16	32	64	128	256	512	1024	C
CP																			
0.02% sol.			0	0	0	0	0	w	s			0	0	0	0	0	w	s	ac
0.01			0	0	0	0	0	tr	s			0	0	0	0	0	tr	s	c
0.005			0	0	0	0	0	tr	w			0	0	0	0	0	tr	s	c
0.0025			0	0	0	0	0	tr	s			0	0	0	0	0	0	s	c
0.0013			w	w	w	w	w	s	s			w	w	w	w	s	s	s	c
0.0006			s	s	s	s	s	s	s			s	s	s	ac	s	ac	ac	c
S																			
0.2% sol.	0	0	s	s	c	c				0	0	w	s	ac	c				c
0.1	0	0	tr	s	ac	c				0	0	0	s	c	c				c
0.05	0	0	tr	s	c	c				0	0	tr	s	c	c				c
0.025	0	0	w	s	c	c				0	tr	s	s	c	c				c
0.013	0	tr	w	s	c	c				w	s	c	c	c	c				c
0.065	tr	s	c	c	c	c				ac	c	c	c	c	c				c
C	ac	c								c	c								

0 = no hemolysis.
tr = trace of hemolysis.
w = weak hemolysis.
s = strong hemolysis.
ac = hemolysis almost complete.
c = complete hemolysis.

Such a two-way test, as shown in Table I, demonstrates the serologic behavior of the two fractions. It is seen in this table that the optimal dilution of antigen—the dilution at which the antiserum shows its full titer—is twenty times as high for CP as for S. The titer of the serum against CP is considerably higher than that against S, and the area of complement fixation differs in shape between the two antigens.

The optimum antigen concentrations of each of the two fractions in the complement fixation test were found to be constant when they were tested against

weak and strong sera, so that these concentrations represent an immunologic characteristic of the respective fractions.

Antibodies to both these fractions were found in all sera of rabbits immunized with whole streptococcal cells. They were found also in the sera of a majority of patients convalescing from acute hemolytic streptococcal infections and of patients with acute or subsiding rheumatic infection. Such antibodies were found also in apparently normal subjects, although generally in a lower range of titer than in the poststreptococcal or rheumatic patients. These data are included in the succeeding paper.

TABLE II

Complement Fixation of CP and S Fractions with Antisera to Other Strains of Group A Hemolytic Streptococcus and to Related Organisms

(*Antigens Derived from NY 5 Strain*)

Strain	Classification	Anti-CP titer	Anti-S titer	Anticomplementary control Serum 1:4
NY 5	Group A	256	64	c
1048 M	" "	64	32	c
1048 M	" "	1024	128	ac
K 151	Group B	<4	<4	c
K 151	" "	8	<4	c
K 151	" "	32	32	c
K 151	" "	32	32	ac
O 90	" "	4	<4	ac
O 90	" "	6	4	c
	Streptococcus viridans	6	6	c
	Smooth pneumococcus	16	32	c
		4	<4	c
		8	16	ac

Breadth of Specificity.—Both the CP and the S fractions gave evidence of broad specificity within group A by reacting with almost all normal human sera, and with almost all sera of patients with streptococcal or rheumatic infections, at their respective ranges of titer. There were also cross-reactions among rabbit antistreptococcal sera of different type-specificity and among anti-CP and anti-S sera of rabbits immunized with fractions derived from streptococci of other types.

In order to detect wider ranges of cross-reaction, complement fixation tests were performed between CP and S prepared from a group A strain and sera of rabbits immunized with group B streptococci, *Streptococcus viridans*, and smooth pneumococci. The results of some of these titrations are shown in Table II. The results show that of each set of non-group A sera at least one

serum reacts with the CP or S derived from group A streptococci, although in a lower range of titer than in the case of group A sera. Both fractions, then, have wide reactivity.

Serologic Distinction of the CP and S Fractions.—The difference between the cytoplasmic particles and the S fraction was indicated by the difference in optimal antigen titer, the shape of the complement fixation area in the optimal proportions test, and the difference in titers of given sera *versus* CP and S. The latter showed considerable variation. The titers of most sera tested were higher against the CP than against S, and the observed difference ranged from a ratio of 16 to 1. As an additional test of the serologic distinction of S from CP, a suspension of the latter was subjected to sonic oscillation and subsequent centrifugation. The supernate of this preparation did not show the serologic characteristics of S, indicating that S was not a product of disintegration of CP.

The final proof of serologic difference is, however, given by the cross-absorption test. In the classical form of the cross-absorption test the immunologic distinction between two antigens is demonstrated as follows: A serum containing antibodies to both antigens is treated with successive aliquots of one antigen until a further addition produces no precipitate. If an addition of the other antigen to this absorbed specimen of serum now produces a specific precipitate, and if the entire experiment can be repeated for another aliquot of the serum with the rôles of the two antigens reversed, the difference between the antigens has been demonstrated. This form of the cross-absorption test was not feasible in the case of CP and S, since repeated additions of each absorbing fraction continued to precipitate small amounts of antibody from antistreptococcal serum. This was probably due to slight contamination of each fraction with the other. Consequently complement fixation tests were used to measure antibodies to each fraction in sera absorbed with various quantities of either preparation. Absorption tests were set up as follows:—

Constant amounts of a given rabbit antistreptococcal serum or of serum from a rheumatic subject were incubated with varying amounts of CP. A similar row of tubes was set up with appropriate concentrations of S. After incubation at 37°C. for 1 hour, and overnight in the refrigerator, each tube was subjected to centrifugation for an hour at 32,000 R.P.M. and the supernate was used in complement fixation tests. A typical absorption experiment is shown in Table III.

This table shows that each fraction, at several concentrations, absorbed from the serum more antibody to itself than to the other fraction.

Immunization Experiments.—The immunization of rabbits with both CP and S fractions was undertaken. In order to simplify the procedure technically, and in view of the difficulty frequently encountered in immunization with isolated bacterial proteins, the technic of Freund and Bonanto (23) was employed.

Each rabbit immunized with CP received 20 mg. of this material dissolved in 1.0 cc. of saline solution. This solution of protein had been emulsified in 1.0 cc. of falba and 4.0 cc. of

mineral oil. The resulting 6.0 cc. of emulsion was injected into two sites on the back of the rabbit. The emulsion of S was treated similarly.

The results of the immunization with CP were quite good. Measurable antibodies appeared within 2 weeks and rose to titers almost as high as those obtained on immunizing rabbits with repeated injections of whole streptococci. These sera were free of anti-S antibodies. In the case of the rabbits immunized with S, antibodies were found to S, although in lower titer than the anti-CP

TABLE III

Cross-Absorption Test of the CP and S Fractions. Human Serum (Rheumatic Fever) Complement Fixation Test

Absorbed with	Tested with												
	CP 1:						S 1:						0
	8	16	32	64	128	256	8	16	32	64	128	256	
CP													
1.6 cc.	c	c	c	c	c	c	s	c	c	c	c	c	c
0.8 "	s	c	c	c	c	c	0	w	c	c	c	c	c
0.4 "	0	s	c	c	c	c	0	w	c	c	c	c	c
0.2 "	0	0	0	s	c	c	0	0	c	c	c	c	c
S													
1.6 cc.	0	w	s	c	c	c	c	c	c	c	c	c	c
0.8 "	0	0	c	c	c	c	s	c	c	c	c	c	c
0.4 "	0	0	w	c	c	c	0	w	c	c	c	c	c
0.2 "	0	0	0	w	c	c	0	w	s	c	c	c	c
O	0	0	0	0	w	c	0	0	s	c	c	c	c

S used in 0.4 per cent solution for absorption.
CP used in 0.8 per cent solution for absorption.

titer of the anti-CP sera. Moreover, it was found that the anti-S sera showed antibodies to CP as well. This was attributed to the known contamination of S by the CP, and to the fact that the latter is more potent antigenically.

Chemical Properties of the Fractions

Analyses for Nitrogen and Phosphorous.—Specimens of CP and S as prepared from several strains of group A streptococci were analyzed for total nitrogen and phosphorus content. Typical results of such analyses, shown in Table IV, show that the nitrogen content of both fractions is consistent with that of protein, and that the percentage of nitrogen in S is higher than that in CP, although some variation is noted from preparation to preparation.

Nucleoprotein Characterization.—Because phosphorus was found in both CP and S preparations, nucleoprotein tests were performed.

The beta-naphthol test was done according to Steudel (24): One cc. of a 0.3 per cent solution of beta-naphthol in concentrated sulfuric acid was overlaid with an equal amount of a 1 per cent solution of the material to be tested, and the tube was agitated slightly. In the case of the CP a deep, reddish brown ring appeared at the liquid interface, whereas preparations of S produced a blue ring.

These two color reactions are characteristic of ribose nucleic acid and of desoxyribose nucleic acid respectively. On further testing, the predominance of ribose nucleic acid in the CP was confirmed by the orcinol reaction and that of desoxyribose nucleic acid in S by the diphenylamine test. Quantitative

TABLE IV

Typical Results of Analysis of CP and S Fractions of Group A Hemolytic Streptococci for Total Nitrogen, Total Phosphorus, and Type of Nucleic Acid

Strain	Preparation	Time of vibration	CP			S		
			N	P	N. A.	N	P	N.A.
		hrs.	*per cent*	*per cent*		*per cent*	*per cent*	
NY 5	5	1	9.5	0.45	RNA*	12.0	1.78	DNA‡
NY 5	8	1	10.8	0.54	"	11.0	1.52	"
NY 5	13	1	11.8	0.70	"	13.0	2.05	"
NY 5	13	4	11.4	0.64	"	12.6	1.85	"
NY 5	14	1	10.3	0.76	"	12.3	1.1	"
NY 5	14	3	9.4	0.59	"	13.0	1.8	"
H 44	4	1	11.5	0.80	"	12.3	2.7	"
H 44	4	4	10.3	0.88	"	11.0	2.5	"
C 203 M	1	1	8.9	0.70	"	10.0	2.1	"
1048 M	3	1	10.9	0.68	"	12.2	2.4	"

* Predominantly ribose nucleic acid.

‡ Predominantly desoxyribose nucleic acid.

analyses of all the CP and S specimens were made by the Mejbaum test for ribose nucleic acid and the Dische reaction for desoxyribose nucleic acid. These will be reported elsewhere (25).

Electrophoresis and Ultracentrifugation.—A number of specimens of both soluble fractions were tested by electrophoresis, and one specimen of each fraction by ultracentrifugation. The CP fraction showed a single boundary by electrophoresis, with a mobility of -4.4×10^{-5} at a pH of 7.4. Since the material was quite opaque the electrophoretic uniformity was determined by examination of both ascending and descending boundaries. A minute contaminant of an electrophoretic mobility corresponding to the chief component of S was noted. In the ultracentrifuge the cytoplasmic particles were shown to be of a very wide range of size. At 19,000 R.P.M. the chamber was cleared in a few minutes, after showing a continuous band of particle sizes across the entire field.

Examination of the S fraction by electrophoresis revealed two major components, presumably protein, one in approximately twice the concentration

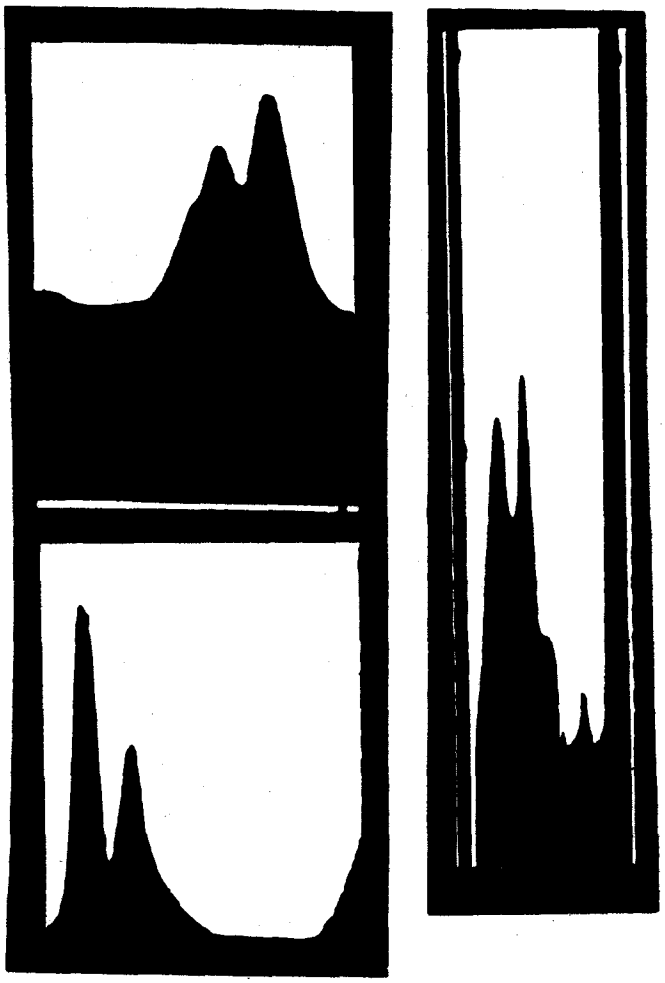

FIG. 1. Electrophoretic (descending and ascending) and ultracentrifugal patterns of a typical preparation of S (strain NY 5). Electrophoresis of a 3.6 per cent solution in phosphate buffer, pH 7.4, $\mu = 0.02$, 25 ma. Ultracentrifugation at same concentration and pH, at a field strength of 170,000 (48,000 R.P.M.).

of the other. These were flanked by a trace of a slower component, which had the mobility of the CP fraction, and a more rapid component, whose mobility was consistent with that of nucleic acid. A typical pattern is shown in Fig. 1. The mobilities of the major components, when the S fraction was tested at a concentration of 3.6 per cent and pH 7.4, are shown in Table V. At a con-

centration of 1 per cent the mobilities were some what higher, at that pH, 7.1 and 5.8, respectively. The same two components, at the same mobilities and relative concentrations, were found on examination of specimens of S prepared from streptococci of strain H 44 and strain 1048.

Ultracentrifugation of S at the same concentration and pH, at a field strength of 170,000 (48,000 R.P.M.) also showed two major components, one of which was approximately at twice the concentration of the other. It was considered probable that the two components were the same by both means of analysis. On this assumption the characteristics of the two components of S are summarized in Table V. The corresponding patterns are shown in Fig. 1. Table V shows quite similar relative concentrations for the larger and smaller protein components of S as determined by the two physical means of examination. On repetition of the ultracentrifugation at approximately one-tenth the concentration used in the earlier run, the two components showed the same sedimentation constants, although the apparent relative concentrations were 86.2 per cent and 13.8 per cent, respectively.

TABLE V

Electrophoretic and Ultracentrifugal Data on the Two Protein Components of the S Fraction

	Electrophoresis		Ultracentrifugation	
	Mobility	Percentage	Sedimentation constant	Percentage
Larger component	−6.7	62.9	4.5	63.3
Smaller component	−5.2	37.1	6.9	36.7

The Structure of the Cytoplasmic Particles.—Analyses of lyophilized preparations of CP were made for lipoids and carbohydrates.

150 mg. of the dried material was extracted three times in a mixture of 25 cc. of ethyl ether and 25 cc. of 95 per cent ethyl alcohol. Each extraction consisted of one-half hour of refluxing on an electric hot plate. The three successive extracts were added and concentrated *in vacuo* to a small volume, which was then divided for determinations of dry weight, nitrogen, and phosphorus.

It was found that the total lipoid extracted by this method ranged from 10.1 to 13.4 per cent. The average nitrogen content of the lipoid extract was 3.4 per cent, and that of phosphorus, 0.5 per cent.

Other aliquots of lyophilized CP were analyzed for the group-specific carbohydrate by the method of Zittle and Harris (6). It was found that 1 per cent of the lyophilized CP was crude carbohydrate. This was consistent with a similar range of yield of carbohydrate from the whole organism, since it will be shown below that the CP is by far the major constituent of the hemolytic streptococcus.

Attempts were made to separate the nucleic acid portion of the nucleoprotein in the CP from its protein by salting out. In contrast to similar experiments performed with S, these attempts were unsuccessful, indicating that the nucleoprotein in CP was a probably true compound with non-polar bonds between protein and nucleic acid.

The Structure of the S Fraction.—The electrophoretic pattern had indicated that the S fraction contained two proteins. Either one or both might be the protein part of the nucleoprotein identified earlier by chemical means. The separation of a component of a mobility consistent with that of nucleic acid in the electrophoretic cell indicated that the nucleoprotein might be of the

TABLE VI

Nitrogen, Phosphorus, and Desoxyribose Nucleic Acid Content of the Dissociation Products of S

Strain	Preparation	Original S		Sediment of ammonium sulfate precipitation		Supernate of ammonium sulfate precipitation	
		N	P	N	P	N	P
		per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
NY 5	3	11.1	2.47	9.6	0.70	9.8	2.38
NY 5	4	13.0	2.05	12.1	0.75	11.8	2.20
NY 5	5	12.6	1.78	12.4	0.13*	12.0	1.75
NY 5	8	12.4	2.80	11.6	0.09	11.8	2.52
NY 5	13	13.2	2.55	12.2	0.64	9.6	2.40
H 44	1	12.1	2.93	12.8	0.80	12.1	2.75
C 203 M	1	10.6	2.86	10.7	1.08	10.0	2.33

* Supernate of two successive precipitations with ammonium sulfate.

dissociable, or protein-nucleate type (26). This hint was confirmed by salting out experiments.

To a solution of S was added three volumes of a cold saturated neutral solution of ammonium sulfate. The redissolved precipitate and its supernate were dialyzed free of ammonium ions, lyophilized, and analyzed for nitrogen and phosphorus.

The results of such experiments, some of which are summarized in Table VI, show that phosphorus was largely lost in the course of the manipulation and that more of it appeared in the supernate, suggesting a dissociation of protein and nucleic acid. More specific evidence of such dissociation was offered by the fact that the desoxyribose nucleic acid content of the supernate was in all cases at least five times as high as that of the sediment. The DNA content thus determined did not account for all the phosphorus found in supernate, since the phosphorus concentration of nucleic acid is 10 per cent, so that phosphorus-containing material other than nucleic acid was present in this fraction. Since the N:P ratio of the nucleic acids is approximately 1.5:1, the relative nitrogen and phosphorus content of the supernate shows that portions other

than nucleic acid were separated from S by the treatment with ammonium sulfate. At least one of the proteins in S is, then, a protein nucleate. It is not possible to say at present whether the other component is of similar nature or is a simple protein.

No attempts to isolate the two protein components of S have been made as yet.

TABLE VII

Typical Relative Yields of R, CP, and S on Varying the Time of Sonic Vibration of Hemolytic Streptococci

Strain	Preparation	Time of vibration	Total dry weight of organisms	R recovered	CP recovered	S recovered
		hrs.	*mg.*	*mg.*	*mg.*	*mg.*
NY 5	13	1	840	420	350	85
NY 5	13	2	820	290	385	87
NY 5	13	3	810	220	650	85
NY 5	13	4	810	90	790	97
NY 5	14	1	500	207	95	54
NY 5	14	1	2100	920	880	127
NY 5	14	2	2200	480	1320	152
NY 5	14	3	2200	160	1560	148
NY 5	14	4	2100	160	1600	164
H 44	4	1	288	72.5	75	84
H 44	4	1	1220	250	292	282
H 44	4	4	1220	85	390	289

The difference between the sum of the fractions and the total weight of organisms is accounted for largely by the four sets of pellets which were known to be mixtures of CP and S, after collecting the pellets included in CP, and to a smaller extent by the dialyzable constituents of the streptococcus.

The Structure of the Hemolytic Streptococcus

The Relation of the Known Antigens to These Fractions.—The presence of the group-specific carbohydrate, or C substance, in the cytoplasmic particle has been noted above. Similar attempts at extraction of the carbohydrate from R (the insoluble residue left by disintegration) and from S failed. It was concluded that the carbohydrate was entirely contained in the cytoplasmic particles.

The type-specific M antigen was obtained from the R fraction by both the Lancefield and Zittle extraction methods. An attempt to use the R fraction itself as an antigen in complement fixation tests for M failed because the R invariably reacted similarly to suspensions of CP. It was concluded that

remnants of CP on the inner surface of the fragments of hulls of streptococci were fixing complement with anti-CP antibodies in the serum. (All anti-M sera available had been prepared by injection of whole organisms.)

The Effect of Variation in the Time of Disintegration.—While studying the effect of varying the physical factors during fractionation, it was found that the yield of CP varied with the length of time of disintegration, whereas that of S remained approximately constant. Typical results of such experiments (Table VII) show that by increasing the time of sonic vibration it is possible to obtain increasing amounts of CP, at the expense of the yield of R. The gross chemical characteristics of the CP are apparently not different in the larger yields than in the smaller. The actual yields are affected by the efficiency of the vibration, which varies among runs, somewhat by the concentration of the organisms, and in at least one observed case, by the strain involved. In the case of strain H 44, the first hour of vibration releases more of the cytoplasmic particles than in the case of other strains, although the final partition of the contents of the streptococcal cell does not vary widely among the strains investigated.

Electron micrographs were taken of the products of sonic vibration of the streptococci. After an hour of vibration, the majority of the cells appear to have lost much of their contents and show partially empty hulls. A few of these hulls have begun to break up into crescentic fragments. After 4 hours of vibration there is marked fragmentation of the hulls of the organisms. Other data reported in this paper indicate that these bits of cell wall have been stripped of almost all the CP which adhered to them at first.

DISCUSSION

The Relation of the New Fractions to Fractions of Streptococcal Nucleoproteins in the Earlier Literature.—Although it is difficult to correlate the results of this physical fractionation with those of chemical extractions, it appears possible that the CP forms the major portion, or all, of the P nucleoprotein of Lancefield, the E-K group of Heidelberger *et al.*, and the NPA of Mudd and Lackman. There is little doubt that the "pigmented heavy particles" derived from sonic extracts of streptococci by Sevag, Smolens, and Stern are the same as the cytoplasmic particles described here. Several differences must, however, be noted. First, the CP described in this paper were white, with no trace of green pigment, except for one group of preparations which showed a faint gray-green cast. It may be that the green pigment of the macromolecules was the result of assimilation of a heavy metal by the streptococci. Again, the speed of centrifugation used in the earlier study to clear suspensions of the macromolecules of heavier impurities was in a range which we found to precipitate considerable amounts of the CP. Finally, the phosphorus content of CP as found in this study is lower than that noted by the earlier authors.

The question arises of the relation of the S fraction to Lancefield's Y and to

Heidelberger's D fraction. In the absence of specimens of those materials it is only possible to say the following: The Y component, which Lancefield encountered but could not isolate, was easily digested by trypsin; forthcoming experiments on tryptic digestion of S may show whether S could be the Y antigen. As for the D fraction of Heidelberger, there are two difficulties in an attempt to identify S with it: the apparently non-polar union of protein and nucleic acid in the D fraction and the fact that the E–K fraction absorbed out antibodies to D in antistreptococcal sera. Both of these findings would not be consistent with the characteristics of S.

The Relation of These Observations to the Total Antigenic Analysis of the Hemolytic Streptococcus.—The structure of the cytoplasmic particles makes it not impossible that antigenic groupings different from those on the surface might be "folded" in the interior of the particle and so not available for reaction *in vitro*. Since the physicochemical means used in resolving the S fraction are not applicable to the CP, other means are being used to investigate its immunologic homogeneity.

The Organization of the Streptococcal Cell.—As a result of much recent work with the constituents of cells other than bacterial, ribose nucleic acid has become associated with nucleoproteins of the cytoplasm and desoxyribose nucleic acid with nuclear elements (27, 28). In the work here reported it was found that the nucleic acid in the CP was predominantly of the ribose type, which is consistent with many observations on cytoplasmic particles derived from other cells. On the other hand, the nucleic acid in S gave the tests for desoxyribose nucleic acid so that it is likely that S contains nuclear material.

These considerations, as well as the inverse relationships between the yield of residue and that of CP, without change in the yield of S, suggest the following hypothesis: It is possible that at first rupture of the cell by the vibration there is fission of the cell wall and of the cytoplasm. The nuclear contents, which are not organized into complex units like those of the cytoplasm, and may be in a more liquid state, pour out in their entirety into the suspending medium. The cytoplasmic particles are then progressively shaken off the fragments of bacterial cells, to form an opalescent stable suspension. What would be called the insoluble residue after an hour of sonic vibration is in reality largely cytoplasmic material. Even after 4 hours of continuous vibration, not all of the cytoplasmic material has been shaken off the fragments of hulls or cell walls of the streptococci, and the inner surface of the latter is lined with cytoplasmic particles. In fact, the R fraction of 4 hour vibration was used twice with success as a substitute for CP in cross-absorption experiments. The truly insoluble residue, consisting of cell walls from whose inner surface all cytoplasmic particles have been stripped might be obtained only after long periods of vibration.

SUMMARY

After disintegration by sonic vibrations the contents of the hemolytic streptococcus can be separated by differential centrifugation into three fractions: an insoluble residue, cytoplasmic particles, and a solution of proteins of smaller unit size.

The residue (R) presumably comprises the cell walls of the bacteria and contains the type-specific M protein. The cytoplasmic particles (CP) contain some lipoid, the group-specific carbohydrate, and nucleoprotein of the ribose type. The supernate fraction (S) contains two components, presumably protein, at least one of which is part of a dissociable nucleoprotein of the desoxyribose type.

Both CP and S precipitate and fix complement with antistreptococcal sera. Both give rise to antibodies on injection into rabbits. Both are of broad reactivity. CP and S can be shown to be serologically distinct by several means, including cross-absorption tests.

On continued disintegration of the organism an inverse relation is noted between the yield of R and that of CP, whereas the yield of S is constant.

A theory as to the structure of the streptococcal cell is offered in terms of the data presented.

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