PROTEIN FRACTIONS OF A SCARLATINAL STRAIN OF STREPTOCOCCUS HEMOLYTICUS*

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As a part of a series of investigations on streptococcal diseases now being carried on in this Department the writers have been attempting to separate and study immunologically active constituents of the scarlatinal type of *Streptococcus hemolyticus*. The work of Hitchcock (1) and the studies of Lancefield (2) have shown that as far as the microbial cell itself is concerned, the protein constituents are the dominating factors, and a study is accordingly being made of these.

The classical method for the isolation of the protein of the bacterial cell involved the use of sodium hydroxide in excess and made little attempt at fractionation (3). It has, however, long been recognized that alkaline solutions can induce profound modifications in native protein, and this is particularly true in the case of the nucleoproteins (4), the group to which the more easily isolated bacterial proteins are believed to belong. Thus Levene (5) extracted dried ground tubercle bacilli with 8 per cent ammonium chloride and found three nucleoproteins in the solution, while Eisler and Silberstein (6) used neutral saline extracts in studying typhoid nucleoproteins as precipitinogens. Also to avoid in part the effect of alkali Johnson (7) proposed to extract the ground, defatted cells first with water, reserving alkaline extractions of the residue for subsequent fractions. This method has been used in the Yale laboratory for the extraction of proteins from members of the tubercle bacillus group, and has been applied to Type III pneumococcus by Stull (8).

In the living microorganism the cell proteins, if not in actual solution, are at least largely present in the form of their salts with bases,

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and this applies particularly to the acidic nucleoprotein group. Native and undenatured cell protein salts should therefore be largely water-soluble, hence the first extraction in the Johnson method might easily remove a mixture which would be difficult to separate.

The method used in the present study was intended to avoid this possibility and at the same time to test the idea that different cell proteins might vary in their acidic strength. It was felt that an effective method of separating such proteins would be to bring them all past their isoelectric points to a degree of acidity which would set them free from the base with which they were combined in the living protoplasm, and then to extract the acidified cellular material successively with buffer solutions of increasing basicity. Thus the strongest protein acid would dissolve in a buffer solution of comparatively low basicity, while a weakly acidic protein would require a more strongly alkaline buffer for its solution.

That the method may be of some utility is indicated by the separation from a scarlatinal strain of *Streptococcus hemolyticus* of a labile, alkali-sensitive, strongly acid nucleoprotein, differing distinctly in its physical and chemical properties, in its specificity, and in its reactivity toward human sera from the fractions obtained by subsequent extraction of the cell residue with more alkaline solutions.

The data given below are to some extent preliminary, but are reported in the hope that the method used might also be of value in the investigation of the proteins of microorganisms other than those being studied in this laboratory.[1]

EXPERIMENTAL

1. Extraction and Isolation of the Acetic Acid Precipitable Protein Fractions.—Strain C_2O_3, a scarlatinal *Streptococcus hemolyticus*, obtained through the courtesy of Mrs. Lancefield, of the Hospital of The Rockefeller Institute for Medical Research, was used since it was reported to contain the type-specific factor. The culture was kept virulent by frequent passages through mice. 50 to 60 l. of a 3 day old culture in proteose-peptone broth were run through a jacketed, steam driven, sterilizable Sharples supercentrifuge designed by Dr. Franklin A. Stevens of this Department to prevent escape of spray. The microorganisms were scraped from the bowl, resuspended in about 400 cc. of saline, and centrifuged. From this point on, all operations were conducted in the cold with chilled reagents unless

[1] Studies on streptococci and the tubercle bacillus group are now in progress.

otherwise specified, the centrifugations, as well, being run in a refrigerating centrifuge (International Equipment Co.) provided with external cooling coils only. The washed cells were suspended in about 400 cc. of acetone, stirred frequently during 2 hours, centrifuged, taken up again in the same quantity of acetone, and allowed to stand overnight. After centrifugation the cells were taken up in 400 cc. of ether which had been freed from alcohol, peroxides, and aldehydes by washing with water, drying with calcium chloride, and storing in the dark over calcium chloride and sodium hydroxide pellets. After frequent stirring during several hours the mixture was centrifuged and the process repeated with a new lot of purified ether. A third lot was finally added and the mixture was allowed to stand overnight, after which it was centrifuged and the organisms were dried *in vacuo*. The yield was about 10 gm. under favorable conditions, about 10 per cent consisting of ash. The acetone-ether extracts were concentrated to dryness *in vacuo* and designated fraction A. The defatted cells were transferred to a ball mill at room temperature and rotated until intact cells could no longer be observed in a smear. The ground material was then rotated in the ball mill with a little 0.2 normal acetate buffer at pH 4 for 10 minutes and rinsed into 5 l. of the same buffer solution and stirred mechanically (all operations in the cold) for 5 to 6 hours, after which the mixture was run through a Sharples supercentrifuge. The effluent, clear after two passages through the machine, contained only 0.125 gm. of nitrogen in the one case in which this was determined, and reacted strongly for specific polysaccharide. It was designated fraction C, and the isolation of the polysaccharide from it will be described later.

The residue in the centrifuge bowl was ground in a mortar with (chilled) M/15 phosphate buffer at pH 6.5, added to 4 l. of the same solution, and stirred mechanically for 6 hours. The mixture was run through the supercentrifuge until clear, chilling as much as possible, and acidified with glacial acetic acid until the resulting precipitate began to flock (fraction D). The residue in the centrifuge bowl was ground in a mortar with water and extracted as before with 3 l. of water, adding enough normal ammonium hydroxide from time to time to maintain the pH at about 8.4, or just pink to phenolphthalein. The mixture was again cleared as well as possible and the effluent cautiously acidified with 50 per cent acetic acid until the precipitate began to flock (fraction E). The slimy residue in the bowl was then treated as before, carrying out the extraction at a pH at which phenolphthalein turned bright red (about 9). The precipitate yielded by the effluent on acidification with normal acetic acid was designated fraction F. The insoluble residue was then given a final extraction either at room temperature at pH 9 to 10, or in the cold at about pH 11 (blue to thymolphthalein), and the acetic acid precipitable material designated G. The generally small amount of insoluble cell residue (fraction N) was set aside for future investigation.

Fraction D was centrifuged off, leaving a clear supernatant at pH 3.8 to 3.9 (bromophenol blue). The Hastings-Sendroy bicolor standard method (9) was used in determining pH in most cases. The precipitate was homogenized in about 200 cc. of water (chilled) and redissolved with the aid of chilled normal

sodium bicarbonate solution which had been neutralized to pH 6.8 by cooling and cautiously adding glacial acetic acid. Care was taken to leave the protein solution faintly acid to litmus at the end. After centrifuging, the small amount of insoluble material was discarded, and the protein solution was diluted to double the volume and reprecipitated with 50 per cent acetic acid. Centrifugation, solution, and reprecipitation were repeated four times, but before the final precipitation the solution was filtered through a 5 inch Berkefeld V candle which had previously been washed through with water containing a little sodium bicarbonate and finally with water. When necessary to produce a clear solution it was again filtered through a Berkefeld N candle. After flocculation of the protein in the above precipitations the supernatants were found to be in the range pH 3.8 to 4.0. The final supernatants and the final precipitate were free from inorganic phosphate. Fraction D was finally washed twice in the centrifuge bottle with water containing a little acetic acid, then twice with redistilled acetone, and was filtered off and dried *in vacuo* over calcium chloride, paraffin, and sodium hydroxide pellets. The yield in favorable cases was 1.4 to 1.5 gm. For analysis a portion was dried to constant weight in a high vacuum at room temperature over phosphorus pentoxide and sodium hydroxide.

Fractions E, F, and G were purified in the same way, using normal ammonium hydroxide for solution in the case of E, and sodium hydroxide in the case of F and G, and never allowing the solution to become more alkaline than about pH 8.5. As before, all operations were conducted in the cold. Supernatants from which E had flocked were usually at pH 4.0 to 4.1, those from F at pH 4.3 to 4.5 and those from G at 4.3 to 4.6. The yields of these fractions varied from 0.05 to 0.4 gm. each.

Each time E, F, and G were redissolved and the solutions centrifuged, particularly when the volume did not exceed 200 to 250 cc., brown, slimy material was deposited. This was collected and saved, and combined with the generally larger amount which collected on the Berkefeld V filter and often made filtration very slow. These slimy residues were ground in a mortar with water up to about 500 cc., made alkaline to about pH 9 (phenolphthalein), stirred thoroughly, and centrifuged for several days in the cold. The residue was treated again as before, until the maximum amount could be dissolved. The supernatants were acidified with acetic acid (to pH 4.6 to 4.8) and the precipitates combined and centrifuged off. Treated as before, very little alkali-insoluble material was deposited, and after a third reprecipitation the solution obtained on redissolving at pH 9 deposited almost nothing on centrifugation and could be run through a Berkefeld filter. The fraction which separated on acidification of the filtrate was designated L.

The supernatants from the original precipitations of C, E, F, and G, and the supernatants from the first two reprecipitations were concentrated to small bulk *in vacuo*, combined, dialyzed in collodion bags, and again concentrated *in vacuo*, made definitely acid with acetic acid, and centrifuged. The supernatant, fraction J, furnished additional specific carbohydrate, while the precipitate, fraction

TABLE I

Properties of Acetic Acid Precipitable Protein Fractions of Streptococcus scarlatinae

Fraction	$[\alpha]_D$	pH of solution used for $[\alpha]_D$ determination	N	P	Basic ash	N:P ratio
			per cent	*per cent*	*per cent*	
7 D	+9.5°	6.2	15.4	3.8	0.9	
F	−44°	7.4	14.9	1.4	0.3	
G	−49°	8.1	14.7	1.0	0.4	
L	−46°	8.4	14.4	1.4	0.3	
D_1	−54°	7.6	15.8	1.2	0.1	
D_2	−46°	6.5	15.1	2.6		
8 D	+12°	6.5	15.2	4.2	0.7	
E	0°	7.7	14.8	4.0	1.5	
F	−25°	9.0	14.2	2.1	0.4	
G	−55°	8.1	14.1	0.8		
H	+22°	7.0	15.8	5.2	1.0	
D_1	−15°	8.2	15.5	3.2	0.5	
D_2	−50°		15.0	1.5(?)		
9 D	+11°	7.9	16.0	3.8	1.1	
E	0°	6.8	14.5	2.8		
F	−42°		14.0	1.1	1.3	
D_1	−64°	8.0	15.4	1.6	0.4	
D_2	−34°	7.7	14.8	2.6		
15 D	0°	7.0	14.6	3.1	0.3	
E	−36°	8.3	15.4	1.9	0.5	
F	−44°	8.2	14.3	1.1	0.6	
G	−41°	7.2	14.0	1.1	1.2	
D_1	−47°	7.8	15.0	1.6	0.0	
D_2	−28°	7.4	16.3	3.3	0.6	
15 D_{3a}	+61°	7.1	15.9	8.9	0.9	1.78
D_{3b}	+19°	7.4	12.1	9.6		
7 D_{3a}	+16°		16.8	7.5		2.24
D_{3b}	+11°		15.4	8.5		1.81

H, was purified and isolated in the same manner as E, omitting filtration through a Berkefeld filter and reprecipitating only once.

Analytical data on the protein fractions are given in Table I. Nitrogen was determined by a slightly modified Pregl micro-Kjeldahl method, phosphorus by the Pregl-Lieb method, and basic ash by decomposing the sample on the water bath with a little nitric acid and igniting at a dull red with nitric and sulfuric acids. The ash was calculated as calcium, and $[\alpha]_D$, N, and P in the table were calculated on the ash-free basis, except in the few cases in which no ash determination could be made.

2. Action of Dilute Alkali on Fraction D.—0.5 gm. of 8 D was dissolved in about 35 cc. of water with the aid of 0.1 normal sodium hydroxide, added drop by drop until the solution remained just pink to phenolphthalein. 6.3 cc. were required. The volume was made up to 50 cc., and 150 cc. of water and 14.3 cc. of 0.1 normal sodium hydroxide were added. After 2 hours 66 cc. of water were added, so that, except for the first 2 hours, in which the concentration of alkali in excess was about N/150, the final concentration of alkali was 0.005 normal. The solution was allowed to stand at 23–26° for 24 hours and was then acidified with acetic acid until the precipitate flocked readily (pH 4.0). After standing overnight in the cold the mixture was centrifuged and the precipitate washed as in preceding examples with water containing a little acetic acid and with redistilled acetone. The amount recovered (D^1) was 0.35 gm. Nitrogen and phosphorus determinations on aliquot parts of the supernatant showed that 15 per cent of the nitrogen and 27 per cent of the phosphorus had been split off. A control portion of 7 D solution which had been diluted to the same relative volume without addition of excess alkali and reprecipitated, showed only 2 per cent of the nitrogen in the supernatant.

The actual amounts of nitrogen and phosphorus in the main supernatant were 11.06, and 5.55 mg., respectively, or approximately in the proportion to be expected if nucleic acid had been split off. The solution was concentrated to small volume yielding a small amount of precipitate on acidification with hydrochloric acid. $[\alpha]_D$ of the precipitate was approximately + 90°.

Since the degradation of fraction D in N/150 to N/200 alkali had caused only a reversal in the direction of optical rotation of the protein and a reduction of 1 per cent in the phosphorus content (see Table I), 0.056 gm. of D_1 was further degraded by dissolving as before in a few cubic centimeters of water and letting stand 24 hours with 0.02 normal sodium hydroxide up to 25 cc. On acidification with acetic acid 0.03 gm. (D_2) was recovered. The product now corresponded in its analytical data and optical rotation to the F and G fractions, except that the nitrogen content was higher. The value for phosphorus is based on a single determination and is probably too high, since duplicate analyses of the supernatant showed 73 per cent of the phosphorus (and 44 per cent of the nitrogen) to have been split off,—again in the ratio approximating that in nucleic acid.

A similar degradation of a mixture of 8 D and 9 D in 0.04 normal sodium hy-

droxide at 25–30°C. resulted in a recovery at pH 4.3 of one-half of the protein as 9 D_1 with a somewhat greater negative rotation than 8 D_2, but otherwise showing similar analytical data. A small fraction, 9 D_2, containing more phosphorus, and evidently hydrolyzed to a smaller extent, was recovered on acidifying the supernatant from 9 D_1 with glacial acetic acid.

Attempts to determine whether or not nucleic acid was split from the protein were made as follows: 1.32 gm. of 15 D were dissolved as in the preceding cases, made up to 100 cc. with water and 20 cc. of 0.1 normal sodium hydroxide, and allowed to stand at 25° for 24 hours. The solution was alkaline to thymolphthalein and alizarine yellow R, but not to orange G or acid fuchsin, so that the pH was between 12 and 14. On acidification to pH 4.6, 0.77 gm. of D_1 fraction was recovered, and on adding acetic acid until the pH was below 3.6, 0.11 gm. of D_2 was obtained, D_1 and D_2 having the same significance as in the case of 9 D (see also Table I). The supernatant was concentrated to dryness *in vacuo*, taken up in a little water, freed from insoluble matter by centrifugation, chilled, and precipitated with 1:1 hydrochloric acid until the solution was acid to Congo red. The precipitate D_{3a} was collected separately and the supernatant treated with several volumes of alcohol, yielding a small amount of additional precipitate (D_{3b}). Each fraction was dissolved in a few cubic centimeters of water with the aid of normal sodium hydroxide solution, reprecipitated with hydrochloric acid, and washed with acidified water and redistilled acetone, using alcohol in addition for the reprecipitation and aqueous washings in the case of *b*. The yields were 0.13 gm. of *a* and 0.02 gm. of *b*. As will be seen from Table I the analytical data on the *a* fraction correspond with those for the nucleic acids (10).

A similar experiment with 7 D yielded analogous fractions, of which the D_{3a} fraction was much lower in optical rotation and somewhat higher in its N:P ratio than was the corresponding fraction from 15 D, the data in this case also being close to those given by the nucleic acids. Possibly it contained more of the levorotatory protein degradation product than did the nucleic acid from 15 D, although the biuret test was negative in a 1:1000 dilution, while it was faintly positive in a 1:200 solution of the 15 D product. When 2 mg. each of 7 D_{3a} and 15 D_{3a} were used for the orcin test, both gave typical pentose reactions, the final amyl alcohol solution showing an absorption band in the orange red.

3. Occurrence of the Type-Specific Factor.—Lancefield (2) was able, by means of acid extraction, to obtain from streptococci from widely different sources type-specific haptens which appeared to be protein degradation products. These were identified by their reaction with sera which had been absorbed by heterologous strains. Aqueous extracts of the organisms also contained type-specific material, but apparently not in antigenic form. Lancefield designated this the M factor.

It appeared of interest to locate, if possible, the type-specific material in the fractions prepared in the course of this work. Type-specific sera were obtained by Lancefield's method, with the aid of a vaccine prepared from the heterologous Strain S 24 of *Streptococcus hemolyticus*, the original culture of which was kindly furnished by Mrs. Lancefield. The results are given in Table IV, according to which fractions 8 H and 7 L contained the specific factor (Lancefield's M substance), in addition to non-specific material. That it possibly exists in antigenic form is indicated by the data on rabbit Serum 75 (Table IV). Two rabbits were injected intravenously three to four times a week for a period of 9 weeks with 0.6 to 1.5 cc. of a solution of 7 L containing 1 mg. per cubic centimeter. Each rabbit received a total of 33 mg. of protein. Bleedings 5 days after the last injection showed the presence of precipitins in the blood of Rabbit 75, and a fraction of these appeared to be type-specific antibodies since they were not absorbed by Strain S 24.

An attempt was also made to shorten the exposure of the streptococcus cells to acetone and ether, but in a case in which the washings were limited to a period of several hours very little material was obtained on extraction at pH 6.5 and its phosphorus content was only 2.7 per cent. Bacilli are known to contain nuclease (11), and it is possible the exposures to acetone and ether used in the case of Preparations 7, 8, and 9, while not too long to cause denaturation of the cell nucleoprotein, nevertheless sufficed to inactivate the nuclease presumably originally present. In Preparation 15 only the acetone extraction was shortened, but since in this case the first aqueous extraction at pH 4 was omitted and the D fraction was extracted with phosphate buffer at pH 7.1 it is uncertain to what cause to ascribe the slightly low rotation and phosphorus of the product and its undoubted contamination with fraction E. However, in Preparation 7 both of the first aqueous extractions were omitted and the initial one was made at pH 8.3. The possibility of simplifying the procedure with these points in mind is now under investigation.

DISCUSSION

It is shown in the description of the experiments that by a preliminary acidification of cautiously dehydrated, mechanically disintegrated scarlatinal *Streptococcus hemolyticus* and subsequent extraction in the cold with phosphate buffer at pH 6.5 it is possible to obtain a nucleoprotein radically different from that prepared according to the classi-

cal method involving alkaline extraction. The new nucleoprotein is characterized by its high phosphorus content, the low pH of its isoelectric point, its rotation of polarized light to the right, and its sensitiveness to alkali. Even in N/200 sodium hydroxide at room temperature the protein loses nucleic acid and becomes levorotatory, the hydrolysis or degradation product being poorer in phosphorus and isoelectric at a higher pH. In N/50 sodium hydroxide three-quarters of the phosphorus is eliminated and the nucleic acid set free may be isolated and identified. Since it shows a strongly positive pentose reaction it belongs to the yeast or pancreas nucleic acid type (10) rather than to that of thymus nucleic acid.

After removal of the nucleoprotein at pH 6.5 successive extraction of the cell residues at pH 8.4, 9, and 10 to 11 yields fractions characterized by their lower nitrogen percentage and poorer phosphorus content, particularly in the last two fractions. These fractions are also the most levorotatory. The three alkaline-extracted fractions appear to differ from each other chiefly in the progressive removal of a common phosphorus-containing component, presumably nucleic acid, with increasing strength of alkali used in the extraction, and differ little in physical properties and analytical constants, except for their lower nitrogen, from the protein fractions obtained by fractional degradation of the neutral-extracted nucleoprotein. An effort is being made to determine whether or not these two groups of fractions are identical.

Serologically the undegraded neutral-extracted nucleoprotein also differs radically from the fractions obtained by alkaline extraction after its removal, and from streptococcus nucleoprotein prepared by the classical method. Thus its reactivity is lower in the precipitin reaction in rabbit antisera obtained by intravenous injection of heat-killed or living bacilli, but it reacts more readily in many cases with the sera of patients suffering from different types of streptococcal disease (Table II). Moreover, the specificity of the neutral-extracted nucleoprotein is different from that of the alkaline-extracted fractions, since on absorption of rabbit antisera it does not remove the antibodies reactive against these fractions. On the other hand, absorption of the sera with the alkaline-extracted fractions removes the precipitins for the neutral-extracted nucleoprotein as well (Table III),

TABLE II

Precipitin Tests with Acetic Acid Precipitable Protein Fractions of Streptococcus scarlatinae

Fraction	Dilution	Rabbit antisera			Fraction dilution	Human sera				Antipneumococcus I, II horse serum
		R1289*	4 (1:1)	41 (1:1)		Ke (Nov.)‡	Ke (Jan.)‡	Ko (Dec.)‡	Ko (Jan.)‡	
7 D	1:20,000	+	−							
F	1:20,000	++±	±							
G	1:20,000	++±	+							
L	1:5000		++							
D_1	1:1000			++						
D_2	1:1000			++±						
					8 D					
8 D	1:5000	+++	±†	±	1:1600	(++++)	(+)	(+)	(++)	
E	1:5000		+±		273§					
F	1:5000	++++	+±		1:1600	(+)	(−)	(−)	(+)	
G	1:5000		+±							
H	1:5000		+							
D_1	1:5000		±							
D_2										
9 D	1:1000		±†	+						
E	1:1000		+							
F	1:1000		+±	+++	9 F					
		R2485*		(1:2)	1:1800					++±
D_1	1:1000	+								
D_2	1:1000	++±		++						

Fraction	Dilution	R1289*	Dilution of fraction	Serum 55	Fraction dilution	Human sera				Antipneumococcus I, II horse serum
						Fe	Br	M	T	
					15 D					
15 D	1:5000	++±	1:1000	+++	1:1000	(++)	(+++)	(+±)	(+++±)	++±
					1800					
E	1:5000	++±								
F	1:5000	++±			15 G					
G	1:5000	++±	1:1000	++++	1:1000	(+)	(+±)	(+±)	(+)	

Readings in parentheses determined after centrifuging at low speed.

* 1:1. Kindly supplied by Mrs. Lancefield.

‡ Data kindly furnished by Dr. Alvin F. Colburn and Miss Ruth Pauli of this Department.

† Turbidity, no whirl.

§ Alkaline-extracted "nucleoprotein" corresponding to F or G. A 1:5000 dilution gave a +++ reaction in Serum 61, 1:1.

TABLE III

Absorption of Sera with Protein Fractions of Streptococcus scarlatinae

Test fractions at 1:1000 dilution. 0.3 cc. sera and fractions, 2 hours at 37°, overnight in the ice box, except as stated below.

Serum absorbed with	Test fraction																	
	7 D			15 D			15 E			15 F			15 G			7 L	8 H	
7 D	±	−	−	+	±	±	+±	++	+++	+±	+±	++	+±	++±	++++	±	+	
15 D	±	−	−	±	±	±	+	+	++	+	±	+±	+	+	+++			
15 E					−			−			−			−				−
15 F					−			−			−			−				−
15 G					−			−			−			−				−

All controls,— except 15G,— saline, ±, ±, ±.

First reading after 2 hours at 37°; second, overnight in the ice box; third reading after centrifugation at low speed. All controls—, except 15G—saline, ±, ±, ±.

Absorption of Serum 61 with Polysaccharide Fraction 8C

Serum absorbed with	Test fraction (1:20,000)						
	8 C	15 D	15 E	15 F			
8 C	−	+++	++++±	++++±			
8 C + 15 D		± (Did not flock)		++ (Flocked)			

a behavior difficult to interpret until after these rather ill defined fractions (E, F, and G, the "nucleoproteins" as ordinarily obtained) have been subjected to more rigorous purification and study. Also, in agreement with Lancefield, the streptococcus proteins encountered in this study precipitate antipneumococcus serum (Table II).

Although absorption of rabbit antisera with specific polysaccharide isolated from fraction C failed to remove the protein antibodies or

TABLE IV

Absorption of Sera with Heterologous Strain S 24 of Streptococcus hemolyticus

Serum	Test fraction							
	9 D	8 D_2	9 E	9 F	7 L	8 H	15 D	15 G
	Dilution							
	1:1000	1:1000	1:1000	1:2000	1:1600	1:1000	1:5000	1:5000
41 (1:2)		+±	++	+±				
41 absorbed (1:2)	−	−	−	−	+	+		
41 (1:1)						++±		
75 absorbed (1:2)					± (+)*	+ (+)	− (−)	− (−)
	Dilution							
				1:2000	1:2000	1:2000		
41 (1:2) after 3 mos.				− (±)	− (−)	± (+)		
R_{1a} (1:1)					+±			

* Values in parentheses obtained after centrifuging at low speed.

alter the differences between them (Table III), in only one instance (8 D) was it possible to absorb with a protein fraction and leave polysaccharide antibodies in the serum.

It is of interest to note that the water-soluble protein fraction obtained by Stull from Type III pneumococcus (8) resembles in its nitrogen and phosphorus content the D fractions summarized in Table I, while the carbonate-extracted fraction is much lower in phosphorus. It is to be regretted that a serological study of these pneumococcus fractions was not reported.

With the aid of type-specific sera prepared according to Lancefield (2) the scarlatinal type-specific factor (Lancefield's M) was found in two fractions, and of these the one which was tested gave indications of containing the material in antigenic form. However, a solution of this fraction, 7 L, 3 months old, appeared to have lost its specific

TABLE V

Degradation of Fraction D by Sodium Hydroxide

Preparation	Concentration NaOH (normality)	Temperature	Time	Total N in supernatant after precipitation with acetic acid	Total P in supernatant after precipitation with acetic acid	Ratio $\frac{\text{Wt. N}}{\text{Wt. P}}$ split off
		°C.	*hrs.*	*per cent*	*per cent*	
7 D	0		—	2		
	0.25	24.5–25	24	77,78		
	0.1	24.5–25	24	66,63		
	0.05		24	59		
	0.01	24.5–25	24	42		
8 D	0.01	24	7	13	22	
	0.01	27–29	24	34		
	0.005	27–29	24	23		
	0.005	23–26	24	15	27	2.0
D_1*	0.02	25	24	44	73	
15 D	0		—	4		
			min.			
	0.1	25	4	17		
			hrs.			
	0.1	25	1	41		
	0.25	37	48		No inorganic P (12)	

* Product of the treatment with 0.005 N NaOH.

reactivity, although the non-specific protein in the fraction seemed unaltered (Table IV). On the other hand, 8 H, kept in a desiccator and dissolved only as needed, has maintained its type specificity for a year.

It is evident from the last experiment reported in Table V that the D fraction cannot be classified as a phosphoprotein in spite of its high

phosphorus content, since inorganic phosphorus is not split off on treatment with 1 per cent alkali (12). Similar tests on an E and an F fraction also gave negative results. Since, however, the phosphorus is split off as nucleic acid from D the new protein belongs definitely to the nucleoprotein group. To consider it as a native cell protein on account of its great lability would be tempting, but premature, for on account of its high phosphorus content it is conceivable that scission from a portion of the originally combined protein had already occurred in spite of the many precautions observed to minimize enzymatic (13) or manipulative hydrolysis. Should the D fraction actually be a degradation product of the original protoplasmic nucleoprotein it would be necessary to classify it as a nuclein, a group of substances ordinarily obtained by peptic digestion of nucleoproteins.

Interesting from the standpoint of nucleoprotein chemistry is the fact that the new protein is not merely a salt of nucleic acid, but contains the nucleic acid in chemical combination. It yields no precipitate with one-third volume of 20 per cent barium acetate solution at pH 6.6 (14), nor are significant amounts of nucleic acid split off until the solution of the protein is rendered alkaline, after which the amount set free depends on the strength of alkali and the period of exposure (Table V). In this streptococcus nucleoprotein, then, the nucleic acid appears to be combined in a labile, ester-like linkage, slowly saponifiable even by alkali as weak as N/200.

The relation of the present work to Lancefield's studies (2) will be clearer when animals have been immunized to the various fractions and the properties of the antisera studied. Until then it may be said that the polysaccharide in fraction C is undoubtedly that designated as "C substance" by Lancefield, while the type-specific factor in fractions 7 L and 8 H is the same as Lancefield's "M substance," except that it appears to be a true antigen instead of a hapten. Fractions E, F, and G would correspond to Lancefield's nucleoprotein P. It is possible that the labile nucleoprotein, fraction D, corresponds to Lancefield's Y, present only in minor amounts in her preparations owing to the use of alkali in extracting the nucleoproteins and the use of hot acid in extracting the type-specific hapten. Should this surmise prove to be correct, Lancefield's Y factor would become known as one of the major antigens of the streptococcus cell.

So far in the present work the isolation of any protein not precipitable by acetic acid has not been attempted, but with the growth of larger amounts of organisms such proteins will be sought for and the study continued of the products already isolated.

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SUMMARY

1. A tentative method is described for extracting a labile nucleoprotein from scarlatinal *Streptococcus hemolyticus*.

2. The product differs chemically and serologically from the fractions prepared by subsequent alkaline extraction of the cell residues, and from protein obtained by the classical method for extraction of bacterial "nucleoproteins."

3. The new nucleoprotein is sensitive to very weak alkalies and readily loses nucleic acid under these conditions. The protein degradation products resemble the alkaline-extracted protein fractions of the cell residues.

4. The bearing of the properties of the new nucleoprotein on the chemistry of nucleoproteins in general is discussed, also the possible relation of the fractions obtained to the analysis of streptococcus antigens made by Lancefield.

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