In any case the data of Table I indicate chondroitin sulfate is higher in young cartilage and decreases with age. Hass reported an increase between ages 2 and 21 followed by a gradual decline. The data obtained in this study confirmed the gradual decline after 21 years, but were insufficient between the ages of 2 and 21 to confirm or fail to confirm the observation of an elevation between these ages. Hass' studies did not include cartilage samples from fetal or of newborns which have a uronic acid content much higher than that of children. When such data are considered, it appears that a high acid polysaccharide level is characteristic of young growing cartilage and may be correlated with active proliferation. A more rapid fixation of sulfate in young tissue has been reported(4,5). This fixation in part is probably a reflection of a higher chondroitin sulfate content.

Conclusions. A study was made of the polysaccharide constituents of cartilage from individuals of various ages. Fetal cartilage was found to be very high in uronic acid. The uronic acid content was strikingly lower by the sixth year and then declined slowly with age. Hexosamine in excess of uronic acid increased with age until maturity was reached after which there was no appreciable change.

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Complement-Fixing Antigens in Concentrates of Streptococcal Culture Supernates.* (21932)

T. N. HARRIS.

From the Rheumatic Fever Research Laboratory, Children's Hospital of Philadelphia, Department of Pediatrics, School of Medicine, University of Pennsylvania.

Recent research on the etiology of rheumatic fever has been directed at the only known association of this disease with any microorganism—association with hemolytic streptococcus. This relationship, which has been reviewed in terms of epidemiologic(1), serologic(2,3) and bacteriologic(4) data, suggested to several investigators that some immunologic relationship might exist between a substance derived from the hemolytic streptococcus and the tissues of the rheumatic host. Within the area of metabolic products of the streptococcal cell there has been considerable study of those antigens which have some biologic or biochemical activity, and to which antibodies can be measured in neutralization tests. Such streptococcal antigens include the hemolysins, hyaluronidase, desoxyribonuclease and kinase (plasminogen activator). Other possible antigens, to which antibodies would be measurable by aggregation phenomena, have not been studied except for 2 studies of hemagglutination reactions with streptococcal supernate concentrates(5,6). The present report concerns an investigation of this potential group of streptococcal antigens.

Methods and materials. Medium. Streptococci were cultivated in either of 2 media, a dialysate medium and, for the most part, in the synthetic medium described by Bernheimer and Pappenheimer(7). The dialysate

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medium was prepared by dissolving dehydrated dextrose broth (Difco) at 20 times the concentration used in preparing ordinary medium and dialyzing this solution in cellophane bags against 19 volumes of distilled water in a rotating dialyzer for 18 hours at 4°C. The pH of the resulting dialysate, (6.5). was brought to 7.2 by addition of molar NaOH. After sterilization in the autoclave. one-tenth volume of sodium bicarbonate was added. This medium, or the synthetic medium, was seeded with an overnight culture of β-hemolytic streptococci, the inoculum being 4% of the volume of the medium. *Concentration of culture supernates.* The combined cultures of each day were pooled, after adjustment of the pH to approximately its original value. distributed into cellophane bags hung on a rack before an electric fan for concentration by pervaporation. After 16 hours of pervaporation at about 12°C, the contents of the bags, now concentrated approximately 10-fold, were pooled and subjected to vigorous centrifugation (30 minutes at 15,000 r. p. m.) to remove bacterial cells. This concentrate was dialyzed overnight against running tap water and then against distilled water, after which the volume was further reduced by pervaporation for 6 hours at 12°C. The pooled concentrates were then desiccated from the frozen state, and the dried material was stored at 4°C. Similar preparations could be obtained by ammonium sulfate precipitation of Seitz filtrates of the cultures but this method produced unsatisfactory yields because of loss of antigenic material by adsorption on the asbestos pad. The strains of β-hemolytic streptococci of Lancefield Group A, were obtained through the courtesy of Dr. Rebecca C. Lancefield. They were maintained in lyophilized form for use in seeding cultures as described above. The human sera obtained from presumably healthy adults in the area of Philadelphia were outdated sera supplied through the courtesy of the Philadelphia Serum Exchange.

Technic of the complement-fixation test. The complement-fixation test was the serologic test used in almost all the work described. When used for estimation of antigenic material, the solution of the latter was prepared in various dilutions, usually in 2-fold steps of dilution, in 0.4-cc volumes. These dilutions were incubated 45 minutes at 37°C with 0.1 cc of a standard serum, diluted to its optimal concentration for this test, and 0.1 cc of guinea pig serum, diluted to contain 1.3 units of complement in this volume. After incubation each tube received 0.1 cc of 4% sheep erythrocytes (adjusted so that 1 cc of the suspension, hemolyzed and diluted to a final volume of 20 cc, yielded a reading of 135 in the Klett photoelectric colorimeter with a #54 filter), and 0.1 cc of rabbit anti-sheep-erythrocyte serum diluted to an optimum concentration for this hemolytic system. For the measurement of antibody the same system was employed, with serial dilutions of serum, and a constant amount of antigen preparation.

Results. A. Production of complement-fixing antigen(s). 1. *Observation of complement-fixing antigens in concentrates of streptococcal culture filtrates.* Concentrates of the supernates of β-hemolytic streptococcal cultures (Group A, type 4, strain H44) in both dialysate and synthetic media were tested by complement fixation in block titration against sera of rabbits, given long courses of injections of killed, and then living streptococcal cells. These rabbits had received streptococci of type 1. Typical results of such complement fixation tests are shown in Table I. It can be seen that a complement fixation reaction is demonstrable between concentrates of streptococcal culture filtrates and rabbit antistreptococcal serum. Antigenic material of streptococcal origin was designated CSA (culture supernate antigens). In exploratory work with these preparations, a small percentage of sera obtained from healthy adults contained complement-fixing antibodies to the streptococcal CSA in a range of titers from those barely measurable by the technic described here up to 32. The relatively higher-titered sera among these were used as reagents for the measurement of CSA, reported below. Table I also shows the results of a complement-fixation test of the same preparations of streptococcal CSA against such a human serum.

Uninoculated synthetic medium was concentrated as described above. The yield of

TABLE I. Complement-Fixation Tests of Streptococcal CSA Produced on Broth-Dialysate Medium and on Synthetic Medium Versus Rabbit Antiserum and Human Serum.

	CS2E (dialysate medium)					CS26 (synthetic medium)					
	Concentration, γ/cc					Concentration, γ/cc					
	250	125	63	31	16	31	16	8	4	2	0
Human serum C											
Diluted: —	0	0	0	1+	2+	0	0	0	2+	2+	2+
1:2	0	0	0	1+	3+	0	0	tr	2+	3+	3+
1:4	0	0	2+	3+	3+	0	2+	3+	3+	3+	3+
1:8	1+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+
Rabbit antiserum 232											
Diluted: 1:8	0	0	0	2+	3+	0	0	tr	2+	3+	3+
1:16	0	0	0	2+	3+	0	0	0	1+	3+	3+
1:32	0	0	1+	3+	3+	0	0	2+	3+	3+	3+
1:64	0	1+	3+	3+	3+	0	3+	3+	3+	3+	3+
—	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+

Degrees of hemolysis: 0, none; 1+, weak; 2+, strong; 3+, complete.

dry weight was 0.4 mg per liter, of the order of one-fortieth the yield of dry weight of CSA per liter of culture. Complement-fixation tests as described above were entirely negative, when this material was used in the same order of concentration as shown for CSA in Table I.

2. *Production of CSA by various types of streptococci of Group A.* To obtain data as to breadth of reactivity of the antigenic material and extent of its production by various types, 6-liter cultures were grown of each type, of a collection of 36 types available. The filtrate of each culture was concentrated, lyophilized and tested for its content of CSA by complement fixation against the rabbit serum mentioned above. In the available strain of each of 5 types, 6, 12, 13, 23, 37, no CSA was found within the threshold of sensitivity of our measurements. In concentrates derived from the other 31 types, CSA was found in a range of concentration from 1 complement-fixing unit per mg of lyophilized material to 16, in the case of our strain of types 25, 30, 36, and 40.

3. *Production of CSA in relation to growth of organisms.* The relationship of concentration of CSA to growth of streptococci was studied as follows: Cultures of several types were seeded into dialysate medium, and at various intervals (0, 4, 8, 12, 24 hours) samples of the well mixed cultures were withdrawn into tubes for immediate centrifugation. The sediment was washed once with saline solution by centrifugation and resuspended in the original volume of the sample for a measurement of turbidity (Klett densitometer, #54 filter). The supernate was dialyzed overnight against running tap water in a ¼ inch cellophane tube and then concentrated by pervaporation to half the original volume. This concentrated culture filtrate was used for the measurement of CSA by complement fixation. In some cultures it was found that the greatest part of the rise in concentration of CSA occurred before the 8th hour of culture, at which time CSA concentration was 1.5 units/cc, and the turbidity of the resuspended bacterial sediment 65; 4 hours later the CSA concentration had risen to 1½ times their value at 8 hours, and the turbidity of the bacterial sediment to 152. Thereafter there was no substantial increase in the value of either quantity. In other cultures it was found that up to 12 hours the increase in the CSA concentration was in general parallel to the increase in the turbidity of the resuspended bacterial sediment, but thereafter the latter increased without corresponding increase in CSA concentration.

B. *Characterization of CSA.* 1. *Effects of pH.* Experiments to determine the effects of high and low pH on activity and solubility of CSA were carried out on solutions of lyophilized concentrates derived from cultures in the synthetic medium. Samples of such solutions were brought to given values of pH

TABLE II. Effect of Previous Incubation with Erythrocytes on Hemolytic and Complement-Fixing Activity of a Preparation of CSA.

	Incubation mixture, 5 ml of 0.4% CSA 82, incubated with														
	A					B					C				
	.6 ml PSS (control)					.6 ml packed RBC and 10 mg $NaHSO_3$					.6 ml packed RBC				
	Tested at dilution of														
	1	2	4	8	16	1	2	4	8	16	1	2	4	8	16
Complete fixation vs. rabbit antiserum No. 1685 at dilutions:															
64	0	0	0	tr	2+	0	0	0	1+	2+	0	0	0	1+	2+
128	0	0	0	tr	2+	0	0	0	1+	2+	0	0	0	1+	2+
256	1+	1+	1+	1+	3+	1+	2+	2+	2+	3+	1+	2+	2+	3+	4+
Hemolytic activity vs. 1 vol. of 0.4% RBC	4+	4+	4+	4+	1+	0	0	0	0	0	4+	4+	4+	1+	1+*

* $NaHSO_3$ added to this preparation for the hemolysin test.
Degrees of hemolysis: 0, none; 1+, weak; 2+, strong; 3+, almost complete; 4+, complete.

by addition of normal hydrochloric acid or ammonium hydroxide, the reagents being added dropwise to the chilled solution with constant stirring. The solutions were kept overnight at 4°C. at the desired pH, after which any precipitate which had formed was removed by centrifugation. Sediments were dissolved in a volume of buffered saline solution equal to that of the original volume, and the pH of the supernate was restored to its original value. Each fraction was then tested for CSA activity by complement fixation, in comparison with the original solution. Between pH 5 and 8.5 there was no precipitation and no loss of activity. At pH 4 a flocculent precipitate was obtained, and the activity of CSA was divided approximately equally between sediment and supernate. At pH 3.5 a more bulky precipitate was obtained, and all of the original activity could be found in the redissolved sediment, no antigen being detectable in the supernate. At pH 1 there was no activity in the supernate, and only half activity could be recovered from the redissolved sediment. At pH 9 a precipitate was obtained, but all serologic activity of the original material could be found in the supernate. At pH 10 a larger, inactive, precipitate was obtained and some loss of activity of the supernate was found. The extent of precipitation of inactive material at pH 9 was not sufficient to increase measurably the activity per mg of the preparation under present conditions of testing.

2. *Precipitation by ammonium sulfate.* A 4% solution of CSA was gradually brought to 50% of saturation with ammonium sulfate by equilibrating the solution with one volume of a saturated neutralized solution of the salt. Additional solid ammonium sulfate was added to saturation. The resulting suspension was neutralized and refrigerated overnight. The sediment was collected by centrifugation on the following day, washed with a saturated solution of ammonium sulfate, and dissolved in distilled water. This solution was dialyzed against running water overnight, in cellophane tubing of ¼" diameter. The supernate was similarly dialyzed. The volume of the redissolved sediment was then brought to the original volume of the solution of CSA, and the redissolved sediment and supernate were tested for complement-fixing activity. The activity of the original solution was completely recovered in the ammonium sulfate sediment, and no activity (less than 3%) appeared in the supernate. Analyses for nitrogen in the case of 3 pools of the CSA as produced in the synthetic medium yielded a mean value of 13.6% N relative to dry weight.

3. *Relation of complement-fixing antigens with other streptococcal antigens.* a. *Hemolysin-O.* The question of the relationship of streptococcal hemolysin-O to the complement-fixing antigens in CSA was approached by using erythrocytes as adsorbents of hemolysin. A solution of CSA, which contained both complement-fixing and hemolytic

TABLE III. Comparison of Complement-Fixation Titers of Various Preparations of Streptococcal Culture Supernates vs. Rabbit Antiserum with Activities of 3 Enzymes in Those Preparations. All values refer to relative concentrations of respective reagents in solutions of culture supernate concentrates at 1 mg/cc.

Preparation	Strain	CF titer	Hyaluronidase titer	Streptokinase titer	Desoxyribonuclease titer	Ratios CF:H	Ratios CF:S	Ratios D:CF
130B	5797	200	<4	16	2000	>50	12.5	10
100A	H105	24	6	24	800	4	1	33
H7	NY5	1	4	<1	64	.25	>1	64
134B	H44	400	4	64	8000	100	6.3	50
H pool	H44	.5	16	<1	96	.03	> .5	192
CS "	H44	400	256	500	6000	1.6	.8	15
C3	H44*	12	96	<1	6000	.13	>12	500
CS 217	RG†	48	1	<1	3000	48	>48	63
CS 224 A	RJ†	32	.5	<1	1500	64	>32	47
CS 225	EC†	24	1	<1	1500	24	>24	63
—	—	—	—	—	—			
				Range of ratios:		>1600-fold	>60-fold	50-fold

* Cultivated in the Chemostat, under constant chemical conditions.
† Fresh human isolation, from non-inflamed pharynx.

activity, was incubated with a suspension of sheep erythrocytes at 4°C, both reagents having been chilled before incubation. After 30 minutes of such incubation erythrocytes were removed by centrifugation in a refrigerated centrifuge, and the supernatant fluid was tested for complement-fixing and hemolytic activity. The results are shown in Table II. It can be seen, on comparison of parts A and B, that the concentration of hemolysin in this preparation has been reduced to less than 6% by the incubation with erythrocytes, whereas the complement-fixing activity of this preparation has been reduced very slightly, if at all. Comparison of the data in part C with that in part B indicates that adsorption of hemolysin to erythrocytes takes place only if the former is in the reduced form. The fact that the very slight loss of complement-fixing activity in preparation B is also found in C would suggest that this loss was not related to the adsorption of hemolysin.

b. *Hyaluronidase, desoxyribonuclease and streptokinase.* Preparations of CSA derived from various cultures were tested for hyaluronidase, desoxyribonuclease and streptokinase activity in simultaneous tests, to obtain data on the association of any of these enzymes with the complement-fixing material. The results of some of these tests are shown in Table III. For inclusion in this Table, preparations were chosen which showed a relatively wide range of ratios of measures of relative activity between complement-fixation and the respective enzymes. Table III shows that the range of ratios of complement-fixation titers to hyaluronidase titers was from 0.03 to >50, and that of streptokinase to complement fixing titers was from 0.8 to >48. The range of ratios of desoxyribonuclease to complement-fixation values was from 10 to 500.

c. *Complement-fixing somatic streptococcal antigens.* In earlier studies the preparation and complement-fixation reactions were described of 2 antigens or groups of antigens derived from extracts of sonically-disrupted streptococcal cells. These were designated as CP (cytoplasmic particles) and S (supernate proteins) (8,9). To obtain data on possible relationships between complement-fixing materials derived in the present study from the culture supernate, and those obtained by disruption of the streptococcal cell, a number of human sera were included in simultaneous complement-fixation tests against all 3 antigenic preparations, each used at its optimal concentration. The results of some of these tests are shown in Table IV. Data are presented in the case of 10 sera which include a relatively wide range of ratios of antibody titers to the 3 antigenic preparations. The range of ratios of anti-CSA to anti-CP titers in respective sera was from less than 0.1 to 2, and the range of anti-CSA titers to anti-S titers was from 0.5 to greater than 16.

Discussion. 1. *Production of complement-*

TABLE IV. Complement-Fixation Titers of 10 Sera Tested with 3 Streptococcal Preparations, CSA and 2 Somatic Fractions, to Illustrate Ranges of Relative Titers.

Serum No.	Titers vs.: CS	CP	S	Ratio of: CS/CP	CS/S
103	96	512	128	.2	.7
3821	12	128	8	.1	1.3
3955	128	96	12	1.3	11
4479	16	256	32	.07	.5
5119	128	192	8	.7	16
5267	128	128	12	1	11
5308	32	96	32	.3	1
5417	64	64	8	1	8
5657	768	384	128	2	6
5717	8	128	8	.07	1

fixing antigens in streptococcal culture. The data presented above would indicate that the substances which give rise to complement fixation with the sera used, are produced by the streptococcal cells in culture. This is suggested by the low yield in dry weight of uninoculated medium concentrated by similar procedures, by the negative complement-fixation tests found with such concentrates of the uninoculated medium, and by the concomitant rise in concentration of complement-fixing antigens in culture supernate with the turbidity of the culture. This antigenic material is broadly reactive, at least within Group A of β-hemolytic streptococci, since cultures of strains of almost all types within this group produced varying amounts of it. That this material, or at least a large part of it, is protein, is suggested by the fact that it is completely precipitable by ammonium sulfate at 80% of saturation, and by the mean nitrogen value of 13.6%. *2. Relation of streptococcal CSA to known streptococcal antigens.* The question of identity of this complement-fixing material or of any components of it with any of the known streptococcal antigens cannot be properly approached until separation of such components has been achieved. In the single case of the O-hemolysin it has been possible to adduce fairly direct evidence that this substance is not responsible for the fixation of complement found with CSA, or at least for a major portion of it, since the adsorption of hemolysin from the crude concentrate by erythrocytes was not accompanied by a decrease in complement-fixing activity. The data on relationship of complement-fixing substances in CSA with other known streptococcal antigens is quite indirect and can be considered only as suggestive. Comparison was made of the concentration of complement-fixing antibodies to CSA with those to somatic nucleoprotein fractions of streptococcal cells to obtain some evidence as to whether the CSA might be largely derived from such cell fractions by dissolution of bacterial cells in culture. The wide range of ratios of antibody titers shown in Table IV would suggest that the fixation of complement by any cellular nucleoprotein which might have been released into the culture medium, could not have accounted for a major portion of the complement fixation in the tests with CSA.

The other group of antigens, examined in part, is that of the extracellular enzymes which, like the CSA, are found in the culture supernatant, and which might as antigenic proteins fix complement with homologous antibodies. While a complete list of such antigens was not examined, in experiments such as those reported in Table III, the wide disparity among measurements of complement-fixing activity, on one hand, and any of the 3 enzymatic activities, on the other, makes it quite unlikely that any of these 3 enzymes are responsible for a major portion of the complement fixation observed. Although there are, of course, other known streptococcal extracellular enzyme-antigens, the number of immunologically distinct antigenic species which would appear to be present in streptococcal CSA according to the data presented in the following paper (at least 7) would render it quite unlikely that the other streptococcal extracellular enzymes could be identical with the respective antigens found in this material. It would appear highly likely, therefore, that an antigen or antigens of streptococcal origin, not identical with any of the known streptococcal antigens, are present in the concentrates of streptococcal culture supernates.

Summary. 1. Supernates of cultures of β-hemolytic streptococci were concentrated with respect to macromolecular material present. Such concentrates gave positive complement-fixation reactions with sera of rabbits which had been given courses of injections of living

streptococci, and with sera of human subjects. 2. Evidence was obtained that complement-fixing antigenic material in such preparations was not identical with the complement-fixing fractions obtained from streptococcal cells, and complement fixation was probably not due to any of a number of the extracellular enzyme-antigens which can be found in streptococcal culture supernates.

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Gel-Precipitation of Streptococcal Culture Supernates with Sera of Patients With Rheumatic Fever and Streptococcal Infection.* (21933)

T. N. HARRIS, SUSANNA HARRIS, AND CLIFTON A. OGBURN.

From the Rheumatic Fever Research Laboratory, Children's Hospital of Philadelphia, the Department of Pediatrics, School of Medicine, University of Pennsylvania, and the Philadelphia General Hospital.

In the literature dealing with antigenic substances produced by the hemolytic streptococcus in liquid cultures the emphasis has been almost exclusively on those antigens which have some biologic activity, such as toxicity or enzymatic function. In present studies the reactions of tissues of the rheumatic host to streptococcal antigens are explored. These studies are concerned not only with streptococcal antigens which have known enzymatic function, but also with those which are demonstrable only by their ability to give rise to antibody formation and to combine with those antibodies in mass reactions such as complement-fixation or precipitation. Production and concentration of macromolecular materials produced in streptococcal culture, and their reaction in complement-fixation tests with sera of injected rabbits and human subjects are described in the preceding paper (1). The complement fixation test does not, however, distinguish between one or more immunologic systems in solution (except in quite special circumstances of wide difference of concentrations of reagents relative to their specific activity). For the purposes of these studies methods of greater resolution were necessary to examine the antigenic materials produced in streptococcal culture. Accordingly, such materials were studied by gel-precipitin tests against rabbit and human sera. During such studies differences were noted in the numbers of antibodies present in sera of various groups of human subjects, in sufficient concentration to be observed. These will be presented here.

Methods and materials. 1. *Antigenic material.* Streptococci of Lancefield group A (Type 4, Strain H_{44}) were cultivated for 18 hours in the synthetic medium described by Bernheimer *et al.*(2). Supernates of centrifugation of such cultures were concentrated by precipitation with ammonium sulfate (at 80% saturation) in the presence of a cellulose fiber filter aid, Solka Floc. The extract of the resulting sediment was collected, suitably

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