

A SEROLOGICAL DIFFERENTIATION OF HUMAN AND OTHER GROUPS OF HEMOLYTIC STREPTOCOCCI

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The differentiation of hemolytic streptococci from various animal sources has been a problem presenting many difficulties. Although numerous biochemical and cultural methods of differentiation have been advanced for the solution of this problem, it is apparent that a satisfactory serological test would be of considerable value. The object of the present paper is to describe a precipitin reaction which differentiates hemolytic streptococci into several groups. The proposed classification is based upon the study of 106 strains of *Streptococcus haemolyticus* isolated from a wide variety of sources: man, other animals, and dairy products. The results of this study are of interest not only from the theoretical viewpoint of establishing an orderly grouping of these microorganisms, but also from an epidemiological aspect in providing a means of identifying the probable origin of a given strain. The biochemical and cultural methods previously employed furnish presumptive evidence of the epidemiological importance and probable source of the strains in question. However, strains are occasionally encountered which are difficult or impossible to classify. Certain of the difficulties inherent in these methods appear to be largely overcome by the use of the precipitin test as a means of differentiation.

Particular attention has been paid by many investigators to the identification of hemolytic streptococci of human origin and to the separation of these strains from those ordinarily associated with bovine mastitis, or normally present in milk and dairy products. Avery and Cullen in 1919 (1) were able to differentiate clearly between one group of strains of bovine origin and those of human origin by determining the final hydrogen ion concentration of cultures grown in 1 per cent

dextrose broth. Ayers and Rupp (2) later showed that the former group was further distinguished by the capacity of its members to hydrolyze sodium hippurate, whereas hemolytic streptococci from human sources did not split this substance.

Brown, Frost, and Shaw (3) concluded that no single test could be relied upon to differentiate the hemolytic streptococci commonly found in milk from the so called *Streptococcus epidemicus*. They were of the opinion, however, that probably the most constant and reliable of the tests tried by them were (a) the final hydrogen ion concentration attained in dextrose broth, and (b) the hydrolysis of sodium hippurate, although apparent exceptions were found to both. Most of the seven groups studied by them were differentiated by these two tests combined with fermentation reactions. They point out, however, that they encountered exceptional strains of bovine origin, characterized by a final pH of about 4.7 in dextrose broth and by little or no hydrolysis of sodium hippurate. These formed an interesting group intermediate between the other strains derived from cattle and those isolated from man. This group had not been described previously.

Streptococci of bovine origin culturally similar to this last group were found in considerable numbers by Minett and Stableforth (4), who concluded that these "streptococci were distinct from the usual udder streptococci and indistinguishable as a group by bacteriological and serological tests from *Streptococcus pyogenes* of direct human origin. The evidence therefore strongly suggests, though it cannot be held to prove, that in these cases the streptococci had been implanted in the udder by the milker." They also state that, although there was an absence of any definite disease among persons in contact with these infected cows, disease does not invariably occur among such persons even when the udder harbors streptococci probably pathogenic for man. These authors, furthermore, give a review of the literature indicating that similar strains were found by J. Smith (5), by Seelemann and Hadenfeldt (6), and by Diernhofer (7), all of whom considered them either closely related to *Streptococcus pyogenes* from man or indistinguishable from strains of human origin.

Edwards (8) described a group of strains from bovine and other animal sources which seemed to possess the same cultural characteristics as those studied by the above mentioned investigators. These strains did not hydrolyze sodium hippurate and were intermediate in their final pH range between the ordinary strains from bovine sources and those of human origin. Among the strains of animal origin studied by him, 94 per cent could be distinguished from those of human origin by the fact that they fermented sorbitol but not trehalose, while the strains from human sources did not ferment sorbitol but fermented trehalose. The remaining 6 per cent of strains derived from animals were, however, similar to the strains from human sources in their fermentation of these substances.

In addition to the streptococci classified by the above authors on the basis of biochemical and cultural reactions, R. C. Avery (9) showed that hemolytic streptococci derived from cheese and similar sources form still another group characterized by the capacity to reduce methylene blue and to grow in a medium containing concentrations of the dye which completely inhibit the growth of strains of human

and bovine origin. Seelemann and Hadenfeldt (6) later called attention to the importance of the size of the inoculum in this test, as some strains ordinarily inhibited in this medium grow in it if a sufficiently large inoculum is used, and, as a result of growth, reduce the methylene blue.

Minett and Stableforth as well as Seelemann and Hadenfeldt, using a test introduced by Belenky and Popowa (10), also showed that the hippurate-hydrolyzing bovine strains grow on blood agar containing 40 per cent bile, whereas human strains and the non-hippurate-hydrolyzing bovine strains only grow to a slight extent on 10 per cent, and rarely on 40 per cent bile blood agar.

Although these and other biochemical and cultural tests have been useful in the differentiation of hemolytic streptococci from various sources, a serological test would add considerable weight to conclusions regarding the origin of a particular strain. The agglutination reaction has heretofore proved unsatisfactory for this purpose on account of (*a*) the troublesome spontaneous agglutination so commonly encountered among streptococci, (*b*) the non-specific cross-agglutination, difficult of interpretation, and (*c*) the existence of so many different specific types as to make identification of strains impractical by type-specific agglutination. In addition to these difficulties, the agglutination reaction has been found inadequate for group classification in the present study because it does not disclose the specific groupings revealed by the results of the precipitin reaction. This lack of parallelism between the agglutination and precipitation tests may, in part at least, be attributable to the distribution within the cell of the various constituents. An insufficient concentration of the specific group antigen at the surface of the bacterial body might prevent the union of this antigen with its antibody with the result that group agglutination would not occur. When, however, the cell constituents are extracted and in solution, the reaction between the group-specific substance and its antibody is no longer masked.

These obstacles can be overcome to a large extent by employing the precipitin reaction. This test is apparently dependent on the presence in streptococci of substances characteristic of the large groups although not specific for the types within the groups. For some years it has been known that hemolytic streptococci of human origin contain a carbohydrate, the so called C substance, which is not type-specific (11); and this was previously thought to be identical in all hemolytic strepto-

cocci. When, however, streptococci from a wider variety of animals were studied, the carbohydrate C, characteristic of human strains, was not found in strains from sources other than man, although it had been found in every strain of human origin previously examined, with a single exception reported by Hitchcock (12). This apparent absence of C from hemolytic streptococci isolated from lower animals led to investigations to determine whether similar substances might be found to differentiate other groups among these strains.

In order to avoid confusion, the existing information concerning the antigenic structure of hemolytic streptococci is summarized.

As far as chemical analysis of the immunologically differentiated substances is concerned, only strains of human origin have been studied previously. The members of this group contain (a) a type-specific protein "M," which differentiates types in agreement with the results obtained by agglutination and mouse protection tests (13 *b, e*); (b) a non-specific nucleoprotein, "P," which gives cross-precipitation and complement fixation reactions with similar fractions from related Gram-positive cocci, namely *Streptococcus viridans*, Pneumococcus, and staphylococcus (13 *a*); (c) another non-type-specific protein, "Y," about which little is known (13 *c, e*); (d) the non-type-specific protein, "Fraction D," of Heidelberger and Kendall (14) which they suggest may be the same as the Y fraction just mentioned; and (e) a non-type-specific carbohydrate, "C" (11, 12), which was formerly thought to characterize hemolytic streptococci and to be common to all strains.

From these cell constituents identified in hemolytic streptococci of human origin, only the carbohydrate C is of special significance in the present study because upon it this classification is based.

Methods

1. Immune Sera.—Rabbits were immunized with formalized cultures as follows: The bacterial sediment from an 18 hour broth culture was suspended in one-twentieth volume of 0.85 per cent sodium chloride solution to which formalin was added in a final concentration of 0.2 per cent. After 48 hours in the ice box these bacterial suspensions were sterile. Immediately before use they were diluted with physiological salt solution to the original volume of the culture. Daily intravenous injections of 1 cc. were given for a week, followed by a week's rest. Two to four series of injections were made. Although good antisera were often obtained after two series, four or more courses were sometimes required. On the 5th day after the last injection test bleedings were made and the serum of animals showing a good titer was collected and stored in the ice box without a preservative.

All strains tried gave usable antisera by this method, although some were better antigens than others. After serial subcultures in 10 per cent type-specific immune

serum, the resulting culture was relatively devoid of type-specific substance and proved the best antigen for inducing the formation of anti-C precipitins specific for each group.

Antisera for the strains of human origin (Group A) were chiefly those which had been prepared previously by a method already described employing increasing doses of heat-killed organisms followed by living culture (13 *c*) although the method noted above was satisfactory for these strains also. With many of the strains of the other groups it was impossible to use a scheme of immunization necessitating the injection of living culture, since too great a loss of animals resulted.

Antisera were tested with extracts of both homologous and heterologous strains of the same group in order to make sure of the presence of the group anti-C precipitin. The type-specific antibody for a subgroup, or type, was often present in addition to the group anti-C precipitin used in this classification, and was sometimes the only antibody present. Consequently if an extract of the homologous strain were the only one used in testing a serum, a type-specific reaction might be obtained which would mask the group, or anti-C, precipitin reaction, and the presence or absence of this anti-C precipitin might not be discovered. Since this classification is based on the anti-C precipitin reaction, it was essential in testing antisera to employ an extract of a strain of heterologous type but homologous group, as measured by the anti-C reaction.

2. *Extracts*.—Extracts were made by a method previously employed in preparing the type-specific substance, M, of strains of human origin (13 *b*). The bacterial sediment from 250 cc. of an 18 hour broth culture was suspended in 5 cc. of physiological salt solution containing sufficient normal hydrochloric acid to make a final concentration of $N/20$ HCl. The reaction of the suspension was tested with Congo red paper and, if necessary, enough hydrochloric acid was added to turn the paper blue. The tube was then immersed in boiling water for 10 minutes, cooled under running water, and centrifuged. The supernatant fluid was neutralized, the resulting precipitate discarded, and the water-clear supernatant fluid was used in the precipitin test. Obviously, such a crude extract contained a mixture of substances, but these did not interfere with the reaction under consideration.

3. *Precipitin Test*.—In performing this test increasing amounts of extract were placed in a series of tubes; usually 0.4, 0.1, and 0.025 cc. constituted the series. The volumes were made up to 0.4 cc. with normal salt solution, and a control tube with 0.4 cc. of the same diluent was included. A constant volume of 0.2 cc. of undiluted antiserum was layered in each tube and allowed to stand for 10 to 30 minutes either at room temperature or in the water bath at 37°C. in order to observe ring formation. The tubes were then shaken and incubated for 2 hours at 37°C. in the water bath. Final readings were made after the tubes had stood overnight in the ice box. Sometimes a larger series of dilutions was employed, but no difference in result was obtained. Extracts from all strains were tested in the same way as those detailed in Table I, but the results of the precipitin test are condensed in Table II and the titration is merely recorded as positive or negative.

4. *Final pH.*—The final pH attained in 1 per cent dextrose broth was determined colorimetrically essentially by the method of Avery and Cullen (1). Readings were made at the end of 4 days' incubation. Some of the first readings were made in duplicate with methyl red and brom-cresol green as indicators, but the later readings were all made with brom-cresol green since with this indicator the color changes were easier to distinguish.

5. *Hydrolysis of Sodium Hippurate.*—This test was made according to the method of Ayers and Rupp (2). The cultures were grown 4 days in infusion broth containing 1 per cent of sodium hippurate; then to 1 cc. amounts of the clear supernatant culture fluid were added 0.3, 0.4, and 0.5 cc. respectively of 12 per cent ferric chloride containing concentrated hydrochloric acid in the proportion of 2.5 cc. per liter. Uninoculated sodium hippurate broth incubated for the same period was used as a control, and sufficient ferric chloride was used in the test to insure complete clearing of the uninoculated control. Care was taken to shake the tubes thoroughly as soon as the ferric chloride was added. If the reaction was positive, a heavy precipitate of ferric benzoate was formed which was insoluble in the excess of ferric chloride. If the reaction was negative, the hippurate and protein precipitates formed at first were redissolved in the ferric chloride leaving a clear solution. Known positive and negative cultures were included as controls in every series tested.

6. *Reduction of Methylene Blue Milk.*—The method of R. C. Avery (9) was used for this test as follows: A 1 per cent solution of methylene blue was added to sterile milk to make a final concentration of 1:5000. 0.1 cc. of fresh 18 hour broth culture was inoculated into 5 cc. of methylene blue milk and into control tubes of milk without the dye. Readings were made after 24 hours' incubation and at intervals up to a week. The 24 hour readings are recorded in the tables, and the few changes occurring later are also noted.

7. *Growth on Blood Agar Containing Bile.*—Rabbit blood agar plates containing 10 or 40 per cent ox bile (10) were used to test the ability of these strains to grow in the presence of bile. Control plates of blood agar without the addition of bile were always inoculated at the same time. The 24 hour readings recorded in the tables were essentially the same as observations made after 3 or 4 days' incubation.

8. *Fermentation Reactions.*—1 per cent trehalose and sorbitol were added respectively to tubes of Hiss serum water with Andrade's indicator. Cultures were observed at intervals, and final readings recorded at the end of a week's incubation (8). Where neither substance was fermented and in case of doubtful growth, subcultures were made on blood agar plates to determine the presence of viable organisms. When tests with both substances were negative, a second set of tubes was inoculated.

9. *Lysis with Streptococcus Bacteriophage.*—These tests were performed by adding 1 cc. of fresh 18 hour blood broth culture (without disturbing the sedimented blood) to 5 cc. of plain broth and incubating for from 2 to 3 hours. To 1 cc. of such a culture 1 cc. of bacteriophage was added. This bacteriophage was derived

from that isolated by Clark and Clark from sewage (15). Either the Berkefeld filtrate of a lysed culture, or the unfiltered culture, crystal-clear after lysis by the phage, was used in these experiments. The only difference observed in filtered or unfiltered reagent was that the filtered phage was somewhat less active. Controls of culture plus broth were also included. The tubes were incubated at 37°C. for from 1 to 18 hours; but lysis was usually complete in 1 hour if it occurred at all. Overgrowth of resistant forms was frequently observed if the tests were incubated overnight.

RESULTS

The specific groupings presented in the tables were made entirely on a serological basis according to the results of the anti-C precipitin test. Table I shows the details of a representative series of precipitin reactions typical of the results obtained with all the strains and recorded in Table II. A control series of precipitin tests with extracts prepared from cultures of *Streptococcus viridans* is given in Table III. The striking group specificity is obvious from Table I. Thus, precipitin tests with extracts of the two representative strains from Group A showed very similar titers with antiserum prepared against another strain of Group A, and did not give any cross-reactions with antisera prepared against hemolytic streptococci of the other four groups represented. The same relationship exists within the other groups: the members of each group react only with serum specific for that group. The correlation between the sources of the cultures and the specific groups into which the strains are differentiated serologically is also made evident in Table I. Group A is composed largely of strains of human origin; Group B is made up of strains derived from mastitis in cows and from normal milk; Group C contains strains from various lower animals; Group D comprises only strains obtained from cheese; and the few strains in Group E were all isolated from certified milk.

Of the 106 strains studied in this series, 104 were classified into five groups, as shown in Tables I and II; two strains in the series remained unclassified. Ten strains of *Streptococcus viridans* included as controls failed to react in any of the group-specific sera. It is reasonable to suppose that with a more extensive survey additional groups may be found, but the number and varied source of the strains studied are sufficient to establish the principle that broad distinct groupings of hemolytic streptococci can be made serologically. No significance can

TABLE I
Specific Precipitin Reactions of Representative Strains from the Five Groups Differentiated by Serological Methods

Cultures					0.2 cc. antiserum prepared against strains of Groups				
Strain	Group	Source	Disease	Extract	A	B	C	D	E*
C 203	A	Man	Scarlet fever	cc.					
				0.4	+++	-	-	-	-
				0.1	++	-	-	-	-
K 96	"	"	Pneumonia	0.025	+	-	-	-	-
				0.4	++	-	-	-	-
				0.1	+±	-	-	-	-
K 107	B	Cow	Mastitis	0.025	-	-	-	-	-
				0.4	-	++	-	-	-
				0.1	-	+++	-	-	-
K 126	"	"	None (certified milk)	0.025	-	++	-	-	-
				0.4	-	++	-	-	-
				0.1	-	+++	-	-	-
P 454	C	Guinea pig	Lymphadenitis	0.025	-	-	++	-	-
				0.4	-	-	+++	-	-
				0.1	-	-	++	-	-
K 150 A	"	Cow	Mastitis	0.025	-	-	+++	-	-
				0.4	-	-	++	-	-
				0.1	-	-	+	-	-
K 158 E	"	Rabbit	Pneumonia	0.025	-	-	++	-	-
				0.4	-	-	+	-	-
				0.1	-	-	++	-	-
K 155 A	"	Horse	Pleuropneumonia	0.025	-	-	++	-	-
				0.4	-	-	+++	-	-
				0.1	-	-	+	-	-
K 155 P	"	Swine	Abortion	0.025	-	-	+++	-	-
				0.4	-	-	+++	-	-
				0.1	-	-	++	-	-
K 155 N	"	Chicken	Slipped tendon	0.025	-	-	+++	-	-
				0.4	-	-	+++	-	-
				0.1	-	-	++	-	-

In the precipitin tests recorded in Tables I, II, and III, all volumes were made up to 0.6 cc. with saline. The tubes were incubated in a water bath at 37°C. for 2 hours and read after standing overnight in the ice box. Controls of serum with saline, and of extract with saline were all negative. The degrees of positive reaction are indicated by + to +++++; a negative reaction is indicated by -.

* The serum of one rabbit immunized with Strain K 129, Group E, gave slight cross-reactions with almost all extracts tested against it. Other rabbits of this series gave negative results as shown.

TABLE I—*Concluded*

Cultures					0.2 cc. antiserum prepared against strains of Groups				
Strain	Group	Source	Disease	Extract	A	B	C	D	E*
K 155 G	C	Fox	Pneumonia	cc.					
				0.4	—	—	++++	—	—
				0.1	—	—	++++	—	—
				0.025	—	—	++	—	—
C 6	D	Cheese	None	0.4	—	—	—	++	—
				0.1	—	—	—	++	—
				0.025	—	—	—	±	—
C 7	"	"	"	0.4	—	—	—	++	—
				0.1	—	—	—	++	—
				0.25	—	—	—	++	—
K 128	E	Cow	None (certified milk)	0.4	—	—	—	—	+++
				0.1	—	—	—	—	++
				0.025	—	—	—	—	±
K 129	"	"	"	0.4	—	—	—	—	+++
				0.1	—	—	—	—	++
				0.025	—	—	—	—	+

be attached to the relative proportions of the several groups since the strains other than those of human origin were chiefly obtained without reference to the frequency of their occurrence.¹

Because the so called anti-C reaction previously studied in members of Group A was found to be due to a carbohydrate, it seems probable that comparable substances of this nature are responsible for the specific reactions in the other groups. The marked group specificity of these substances is apparently an important factor in the notable lack of cross-reactions among groups. Another factor is the nature

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TABLE II a
Serological Grouping and Summary of Biochemical and Cultural Characteristics of All Strains
Group A: Strains Chiefly of Human Origin

Strain	Origin of cultures		Serological reactions				Biochemical and cultural reactions							
	Source	Disease	Precipitin tests				Final pH in dextrose broth	Hydrolysis of sodium hippurate	Reduction of methylene blue milk	Growth on bile agar		Fermentation		Lysis with streptococcus bacteriophage
			A	B	C	D				10 per cent	40 per cent	Trehalose	Sorbitol	
1	N. Y. 5	Human	Scarlet fever	+	+	+	+	5.0	-	++	+	+	-	-
2	C 203	"	"	+	+	+	+	5.0	-	++	+	+	-	-
3	O 89	"	"	+	+	+	+	4.8	-	+++	+	+	-	-
4	K 151 D	"	"	+	+	+	+	5.1	-	+	+	+	-	+
5	K 151 E	"	"	+	+	+	+	5.1	-	+	+	+	-	+
6	K 96	"	Pneumonia	+	+	+	+	5.0	-	+	+	+	-	+
7	K 138	"	"	+	+	+	+	5.0	-	+	+	+	-	-
8	K 139	"	"	+	+	+	+	5.0	-	+	+	+	-	-
9	K 148	"	"	+	+	+	+	5.2	-	+	+	+	-	-
10	K 169	"	"	+	+	+	+	5.0	-	+	+	+	-	-
11	K 109	"	Infected gland	+	+	+	+	5.0	-	+	+	+	-	+
12	K 152	"	Lung infarct	+	+	+	+	5.0	-	+	+	+	-	-
13	K 197	"	Menigitis	+	+	+	+	5.2	-	+	+	+	-	-
14	M 34	"	Tonsillitis	+	+	+	+	5.0	-	+	+	+	-	-
15	K 210	"	Peritonsillar abscess	+	+	+	+	5.2	-	+	+	+	-	-
16	Boston	"	Septic sore throat	+	+	+	+	5.0	-	+	+	+	-	+
17	K 151 B	"	"	+	+	+	+	5.0	-	+	+	+	-	-
18	K 151 C	"	"	+	+	+	+	5.0	-	+	+	+	-	+
19	K 171 E	"	"	+	+	+	+	5.0	-	+	+	+	-	+
20	K 171 F	"	"	+	+	+	+	5.2	-	+	+	+	-	-
21	K 171 G	"	"	+	+	+	+	5.2	-	+	+	+	-	-
22	K 158 C	"	Spontaneous infection	+	+	+	+	5.1	-	+	+	+	-	-
23	V 10†	Rabbit Bovine	Mastitis (epidemic of septic sore throat)	+	+	+	+	5.1	-	+	+	+	-	-

of the crude extracts employed which, although composed of a mixture of active substances, nevertheless contained little or none of the protein fractions, such as P, because, by the method of extraction, most of the acid-precipitable and heat-coagulable proteins are removed from solution. In addition, the antisera were prepared in a manner calculated to prevent the formation of much protein antibody. Consequently, even with such a complex antigenic mosaic, only a minimal number of cross-reactions was observed. These occurred as disc precipitates and were usually so slight as almost to escape detection. The major reaction was generally strong enough to make the proper classification evident even without the confirmatory evidence of the biochemical reactions. Further work is necessary before the nature of this disc-like cross-reaction can be ascertained. The few exceptions to the group specificity commonly observed are indicated in Table II and are considered in detail in the discussions of the groups in which they occur.

In addition to a summary of the precipitin reactions, all of which were performed in the same manner as the tests recorded in Table I, Table II contains results showing the correlation between the serological reactions and certain of the cultural and biochemical reactions found of value by other investigators in differentiating hemolytic streptococci. The marked susceptibility to lysis by bacteriophage, exhibited by members of the two groups, C and E, and previously reported (16), is also recorded in Table II. The results of all these tests were used as confirmatory evidence of the validity of the present

Footnotes to Table II a

In Table II, + indicates a positive reaction, and - indicates a negative reaction. In the precipitin tests and the test for the hydrolysis of sodium hippurate, this is a condensed form and represents a titration using several dilutions. The degree of growth on bile agar is represented by + to +++++. The details of the performance of all tests are given under Methods.

* Growth and reduction after 3 days' incubation.

† Isolated from healthy individuals during an epidemic of scarlet fever and septic sore throat in which K 151 D and K 151 E were obtained. All four are of the same serological type as Strain C 203 (22).

‡ Obtained from Dr. O. T. Avery. Considered to be a human strain by Avery and Cullen (1) as well as by Smith and Brown (17) who isolated it during an epidemic of septic sore throat.

classification based on the anti-C precipitin reaction. The characteristics of the groups defined are the following:—

Group A.—Extracts of the 23 strains placed in Group A gave positive precipitin reactions with antisera prepared against any member of the group, and gave no cross-reactions with antisera for any other group. The classification made on this basis was confirmed by additional biochemical and cultural tests. The strains of Group A, with two exceptions, were all derived from human sources.

One exceptional strain, V 10, came from the udder of a cow suffering from mastitis and was isolated during one of the Massachusetts epidemics of septic sore throat by Smith and Brown (17), and studied later by Avery and Cullen (1). Both groups of authors considered it a strain primarily of human origin and thought that the cow had probably become infected through a milker. The other strain in Group A, not of human origin, was isolated from a spontaneous lung infection in a rabbit. Whatever may be the true explanation of such exceptions, it is obvious that an overwhelming majority of strains in Group A are of human origin.

Turning now to groupings made on the basis of biochemical and cultural characteristics, quite good agreement with the classification by serological means is found.

When judged by the two tests principally recommended by Brown, Frost, and Shaw, namely final pH in dextrose broth and hydrolysis of sodium hippurate, it is seen that all the strains of Group A reacted with respect to these tests in the manner characteristic of strains of human origin. One strain gave a final pH of 4.8, while the rest ranged between pH 5.0 and 5.2, and none hydrolyzed sodium hippurate. None reduced methylene blue milk within 24 hours, although two strains gave growth and evidence of reduction after 3 days' incubation. These two exceptional results may have been dependent on the size of the inoculum, a factor stated (6) to be of considerable importance in the reaction. A few strains of this group grew on 10 per cent bile blood agar, and one even showed slight growth on 40 per cent bile blood agar. Minett and Stableforth (4) also found a few strains isolated from human sources with which growth was not inhibited on this medium.

The differential fermentation of trehalose and sorbitol was tested since it was advocated by Edwards (8) as a means of distinguishing between hemolytic streptococci of human derivation and those strains of animal origin which resemble human strains in not hydrolyzing sodium hippurate. All the members of Group A fermented trehalose and none fermented sorbitol, which is in agreement with Edwards' findings.

Of the 23 strains in Group A only six were found partially susceptible to the action of streptococcus bacteriophage and not very markedly so.

Group B.—The 21 members of Group B were classified together on the basis of their common precipitin reactions. The only cross-

TABLE II b
Serological Grouping and Summary of Biochemical and Cultural Characteristics of All Strains
Group B: Strains Chiefly of Bovine Origin

Origin of cultures		Serological reactions				Biochemical and cultural reactions								
Strain	Source	Disease	Precipitin tests				Final pH in dextrose broth	Hydrolysis of sodium hippurate	Reduction of methylene blue milk	Growth on bile agar		Fermentation		Lysis with streptococcus bacteriophage
			A	B	C	D				10 per cent	40 per cent	Trehalose	Sorbitol	
1	V 8	Bovine	-	+	-	-	4.4	+	-	+++	+++	+	-	-
2	V 9	"	-	+	-	-	4.4	+	-	+++	+++	+	-	-
3	C 69	"	-	+	-	-	4.5	+	-	+++	+++	+	-	-
4	K 107	"	-	+	-	-	4.4	+	-	+++	+++	+	-	-
5	K 151 A	"	-	+	-	-	4.4	+	-	+++	+++	+	-	-
6	B 63	"	-	+	-	-	4.5	+	-	+++	+++	+	-	-
7	B 92	"	-	+	-	-	4.4	+	-	+++	+++	+	-	-
8	B 112	"	-	+	-	-	4.4	+	-	+++	+++	+	-	-
9	B 115	"	-	+	-	-	4.6	+	-	+++	+++	+	-	-
10	B 116	"	-	+	-	-	4.4	+	-	+++	+++	+	-	-
11	B 120	"	-	+	-	-	4.5	+	-	+++	+++	+	-	-
12	B 125	"	-	+	-	-	4.5	+	-	+++	+++	+	-	-
13	B 126	"	-	+	-	-	4.4	+	-	+++	+++	+	-	-
14	B 132	"	-	+	-	-	4.4	+	-	+++	+++	+	-	-
15	B 135	"	-	+	-	-	4.6	+	-	+++	+++	+	-	-
16	M 216	"	-	+	-	-	4.5	+	-	+++	+++	+	-	-
17	K 126	"	-	+	-	-	4.6	+	-	+++	+++	+	-	-
18	K 127	"	-	+	-	-	4.5	+	-	+++	+++	+	-	-
19	K 198*	"	-	+	-	-	4.6	+	-	+++	+++	+	-	-
20	O 90†	Human	-	+	-	-	4.5	+	-	+++	+++	+	-	-
21	K 158 A	Rabbit	-	+	-	-	4.4	+	-	+++	+++	+	-	-

* The child from whom this culture was obtained had always drunk unpasteurized certified milk.

† Obtained as Aronson scarlet fever strain but different from another strain (O 89) labelled "Aronson" which was furnished from another laboratory.

reactions between members of this group and antisera for other groups were a few slight disc-like precipitates observed with extracts of two strains.

Strain M 216 gave a cross-precipitin test with Group C antiserum in addition to the much stronger major reaction with Group B antiserum. Strain K 158 A gave a cross-reaction with Group D antiserum, but here again the reaction with Group B antiserum was much more marked. In both instances all other characteristics were typical of Group B. The strains of this group were all similar in their biochemical and cultural characteristics. They fermented dextrose in broth to a final pH of 4.4 to 4.6; all hydrolyzed sodium hippurate; they did not reduce methylene blue milk; they grew actively on blood agar containing even 40 per cent bile; they were similar to strains of Group A in fermenting trehalose but not sorbitol; and they were not susceptible to streptococcus bacteriophage.

Group B, comprising 21 strains chiefly of bovine origin, corresponds to the group called *Streptococcus mastitidis* by many authors. In this series two strains isolated from man and one from a rabbit also fell into this group on the basis of the precipitin test, a finding confirmed in all three instances by the cultural and biochemical characteristics. One of these, Strain K 198, was obtained in almost pure culture from the throat of a child who had been exposed to scarlet fever. Neither at the time of taking the throat culture, nor subsequently, did she develop any symptoms of the disease, and it was ascertained that she had always drunk unpasteurized certified milk.

There is some uncertainty concerning the source of Strain O 90, another exceptional member of this group, in that it also has a history of human origin. Dr. E. W. Todd, who furnished the culture, states (18) that it is an encapsulated streptococcus coming originally from Dr. Wamoscher as one of Aronson's scarlatinal strains. Dr. Todd also sent us the strain "Aronson Schnitzer," with the note that it was entirely different from the other Aronson strain. "Aronson Schnitzer" (O 89 in our series) fell into Group A, while "Aronson Wamoscher" (O 90 in our series) had all the characteristics of Group B. Strain O 90 could, in fact, be identified as a member of this group both by its cultural and biochemical characteristics and by the specific precipitin reaction characteristic for this group.

No. K 158 A, the third strain in this group not isolated from cattle or milk, was obtained from a rabbit which had been used in the laboratory for testing vaccine virus.

Group C.—The members of Group C were derived from a variety of animal sources other than man. This group of 49 strains distinguished by the specific anti-C precipitin reaction, is largely composed of strains which have, by other methods, been difficult, or at times impossible, to differentiate from hemolytic streptococci of human origin. By

means of the precipitin reaction, however, the specific differentiation of this group is unequivocal. The single cross-reaction observed was a slight disc-like precipitate formed when an extract of Strain K 106 was tested with certain antisera for Group B, but not with all antisera.

All of the 18 strains isolated from guinea pigs belonged in this group together with most of those from rabbits, the majority of the strains of bovine origin which did not hydrolyze sodium hippurate, and all strains originating from diseased horses, foxes, swine, and chickens. The strains from the last four sources, as well as a number from cattle, were kindly supplied by Dr. P. R. Edwards. Streptococci of bovine origin which have been described as having cultural characteristics very similar to those of the strains in this group have caused several English and German investigators (4) to express doubt concerning the possibility of distinguishing them from strains of known human origin, although Dr. Edwards was able to differentiate 94 per cent of those he examined by means of the fermentation of two substances. By comparing the cultural and biochemical characteristics of this group with those of Group A it is easy to understand the possible difficulty in deciding definitely on the basis of these tests that strains of Group C are not of human origin. The members of this group had a final pH range in dextrose broth of 4.6 to 4.9, which was intermediate between the ranges of Groups A and B, and indeed, overlapped to some extent the pH ranges of these two groups. The strains of Group C were also similar to those of Group A in the following respects: they did not hydrolyze sodium hippurate nor did they usually reduce methylene blue in milk, although a few exceptions were observed after 48 hours' incubation; and only one strain grew on 40 per cent bile blood agar, but most of them grew more or less abundantly on blood agar containing 10 per cent bile. Forty-five of the 49 strains in this group fermented sorbitol and not trehalose, a characteristic distinguishing them from streptococci of human origin. Four strains did not ferment either of these substances. Two of these, K 155 B and K 196, were *Streptococcus equi* according to Holman's classification (19); and Edwards found that among the streptococci examined by him only *Streptococcus equi* failed to ferment either sorbitol or trehalose. In the present series, two strains isolated from guinea pigs (Strains P 230 and K 104) also left these substances intact, but differed from *Streptococcus equi* in fermenting lactose.

As reported in a previous paper (16), the majority of hemolytic streptococci susceptible to the bacteriophage originally isolated by Clark and Clark (15) were found in this group; and only two strains included in the group were not subject to lysis in these experiments. Of these, K 196 was tested on one occasion only, but K 155 L was tested repeatedly under the most favorable conditions. With this culture it is possible that resistant forms had entirely overgrown the non-resistant. This was suggested partly by the fact that growth of the culture on blood agar plates always occurred in the form of fine, dry colonies without mucoid characteristics. This growth was similar to that of a resistant subculture of the original susceptible strain (R 226).

TABLE II C
Serological Grouping and Summary of Biochemical and Cultural Characteristics of All Strains
Group C: Strains Isolated from a Variety of Lower Animals

Origin of cultures		Serological reactions				Biochemical and cultural reactions								
Strain	Source	Disease	Precipitin tests				Final pH in dextrose broth	Hydrolysis of sodium hippurate	Reduction of methylene blue milk	Growth on bile agar		Fermentation		Lysis with streptococcus bacteriophage
			A	B	C	D				10 per cent	40 per cent	Trehalose	Sorbitol	
1	K 56	Lymphadenitis	-	-	+	-	4.7	-	-	++	-	-	+	+
2	K 57	"	-	-	+	-	4.8	-	-	+	-	-	+	+
3	K 58	"	-	-	+	-	4.8	-	-	+	-	-	+	+
4	K 59	"	-	-	+	-	4.9	-	-	+	-	-	+	+
5	K 60	"	-	-	+	-	4.8	-	-	+	-	-	+	+
6	K 61	"	-	-	+	-	4.8	-	-	++	-	-	+	+
7	K 62	"	-	-	+	-	4.7	-	-	+	-	-	+	+
8	K 64	"	-	-	+	-	4.8	-	-	±	-	-	+	+
9	P 230	"	-	-	+	-	4.7	-	-	±	-	-	+	+
10	K 104	"	-	-	+	-	4.8	-	-	++	-	-	+	+
11	K 159	"	-	-	+	-	4.8	-	-	++	-	-	+	+
12	K 132	"	-	-	+	-	4.8	-	-	++	-	-	+	+
13	P 454	"	-	-	+	-	4.7	-	-	++	-	-	+	+
14	P 546	"	-	-	+	-	4.8	-	-	++	-	-	+	+
15	K 171 A	"	-	-	+	-	4.8	-	-	+	-	-	+	+
16	K 171 B	"	-	-	+	-	4.7	-	-	+	-	-	+	+
17	K 171 C	"	-	-	+	-	4.8	-	-	+	-	-	+	+
18	K 171 D	"	-	-	+	-	4.6	-	-	+	-	-	+	+
19	K 106	Mastitis	-	±	+	-	4.7	-	-	++	-	-	+	+
20	K 150 A	"	-	-	+	-	4.9	-	-	++	-	-	+	+
21	K 150 B	"	-	-	+	-	4.8	-	-	+	-	-	+	+
22	K 150 C	"	-	-	+	-	4.8	-	-	+	-	-	+	+
23	K 150 D	"	-	-	+	-	4.8	-	-	++	-	-	+	+
24	K 150 E	"	-	-	+	-	4.8	-	-	+	-	-	+	+

The three remaining cultures highly susceptible to bacteriophage formed Group E; but a few considerably less susceptible strains were included in Group A, so that this test was not specific for any serological group or groups, although its close association with Groups C and E was noteworthy.

One series of cultures in this group, supplied by Dr. F. H. Fraser of the University of Toronto, is of particular interest and illustrates how useful this test may be. Strains K 171, A, B, C, and D, isolated from guinea pigs in a spontaneous epidemic caused by hemolytic streptococci, all proved to be members of Group C; while Strains K 171 E, F, and G, isolated at that time during routine examinations of the throats of healthy attendants, all fell into Group A, the group containing the larger majority of strains from human sources.

Group D.—Group D, a uniform group of eight strains all derived from cheese, were among those used by Avery and Cullen (1) in their study of the final hydrogen ion concentration attained by hemolytic streptococci.

These strains attained a final pH in dextrose broth of 4.2 to 4.3; they hydrolyzed sodium hippurate, but only to a slight extent; they grew readily in methylene blue milk and reduced it overnight; they grew luxuriantly on blood agar containing as much as 40 per cent bile; they were variable in their fermentation of trehalose and sorbitol, although six of the eight strains examined fermented both; and they were not susceptible to lysis by streptococcus bacteriophage.

Group E.—Group E comprised three strains isolated by Dr. J. H. Brown from certified milk. They were members² of Groups 3 and 6 described by Brown, Frost, and Shaw (3). On preliminary examination they were thought to be members of Group C of this series because one of them, K 131, showed some cross-reaction with Group C antisera and because all three were very hemolytic on blood agar plates and also markedly susceptible to streptococcus bacteriophage. Further work showed that the precipitin reaction of Strain K 131 with Group C antisera was a minor one, not exhibited by the other two strains, and that antisera prepared against these three strains showed strong precipitin reactions with extracts of all three.

The antiserum from one rabbit immunized with Strain K 129 showed traces of cross-reactions with almost all extracts tested. This was not evident with the antiserum from another rabbit; hence it was felt that this difference had to do with an individual rabbit variation rather than with antigens contained in this strain.

² See footnote (*) to Table II *d*.

TABLE II d
*Serological Grouping and Summary of Biochemical and Cultural Characteristics of All Strains
 Group D, Group E, and Unclassified Strains*

Strain	Origin of culture		Serological reactions					Biochemical and cultural reactions							
	Source	Disease	Precipitin tests					Final pH in dextrose broth	Hydrolysis of sodium hippurate	Reduction of methylene blue milk	Growth on bile agar		Fermentation		Lysis with streptococcus bacteriophage
			A	B	C	D	E				K 130	10 per cent	40 per cent	Trehalose	
Group D: strains isolated from cheese															
1	C 1	Cheese	-	-	-	+		4.2	±	+	++++	+++	+	+	-
2	C 2	"	-	-	-	+		4.2	±	+	++++	+++	+	+	-
3	C 3	"	-	-	-	+		4.3	±	+	++++	+++	+	+	-
4	C 4	"	-	-	-	+		4.3	±	+	++++	+++	+	+	-
5	C 5	"	-	-	-	+		4.3	±	+	++++	+++	+	+	-
6	C 6	"	-	-	-	+		4.2	±	+	++++	+++	+	+	-
7	C 7	"	-	-	-	+		4.2	±	+	++++	+++	+	+	-
8	C 8	"	-	-	-	+		4.2	±	+	++++	+++	+	+	-
Group E: three strains isolated from certified milk															
1	K 128*	Bovine	-	-	-	+		4.8	-	-	-	-	+	+	+
2	K 129	"	-	-	-	+		4.6	-	-	-	-	+	+	+
3	K 131	"	-	-	-	±		4.6	-	-	-	-	+	+	+
Unclassified strains: one of bovine origin, one of human															
1	K 130	Bovine	±	±?	-	-		4.6	+	+	+++	+	+	+	-
2	K 208	Human	-	-	-	-		4.8	-	-	-	-	+	+	-

* K 128 of this series is "Certified Milk 21," Group 6, of Brown's series (3); K 129, "Certified Milk 10," Group 3, of Brown's series (3); K 131, "Certified Milk C 3056," Group 3, of Brown's series (3).
 In addition to these strains, Dr. Brown kindly sent me "Certified Milk 4," Group 2, and a strain representing his Group 7, "Certified Milk 22-76" (Strains K 126 and K 127 respectively of this series), both of which fell into Group B serologically and culturally.
 Dr. Brown's strain, "Certified Milk 19," Group 5, is in this series an unclassified strain, K 130.

The cultural characteristics of this group were the following: the final pH attained in dextrose broth was 4.6 to 4.8; sodium hippurate was not hydrolyzed; methylene blue milk was not reduced; growth was not obtained on bile blood agar even when the concentration of bile was only 10 per cent; both trehalose and sorbitol were fermented; and the streptococcus bacteriophage caused rapid lysis of all three strains. It is not known how extensively this group is distributed, nor whether it is composed entirely of strains derived from milk, although the three classified here were isolated from certified milk.

Unclassified Strains.—Two strains were not classifiable in any of the above groups.

One of these, Strain K 130, from certified milk, was very similar culturally to the strains isolated from cheese. It was not as high an acid producer as the latter, reaching a final pH of only 4.6 in dextrose broth; in all other cultural and biochemical characteristics it was similar to members of Group D. Serologically, however, it was quite distinct. An extract prepared from a culture of Strain K 130 did not precipitate Group D antisera; and antisera prepared against Strain K 130 reacted with it alone out of the 106 strains in this series. A strong cross-reaction was observed between extracts of this strain and two antisera against strains of Group A, although other antisera prepared against the same strains at the same time showed no trace of cross-reaction. The homologous K 130 antiserum gave, however, the strongest reaction of all.

The other unclassified strain, K 208, of human origin, showed no hemolysis on the surface of blood agar plates until after 2 or 3 days' incubation. Deep colonies, however, showed definite zones of hemolysis after 18 hours' incubation, leaving no room for doubt that it was a beta hemolytic (21) type of streptococcus. This strain was isolated from a tooth abscess, and culturally was similar to the strains of Group A, although serologically it was different from all the strains for which antisera were available. No antiserum for this strain was prepared.

Streptococcus viridans.—Table III shows the results of the precipitin reactions of ten strains of *Streptococcus viridans* which were used as controls.

One of these, Strain K 157, was isolated from a guinea pig infection by Dr. Theobald Smith (20) and designated "Strain C" in his series, and the rest were from patients in hospitals. Strain A 84 showed a slight cross-reaction with a Group B antiserum, and Strains 38 D and K 157 showed a trace with a Group D serum, but all others tested were negative.

Other Cultural Characteristics.—In addition to the characteristics of the hemolytic streptococci listed in Table II certain others were observed.

The degree of hemolysis was fairly characteristic for each group. Thus, the members of Group B were the least hemolytic (about +), those of Group A considerably more hemolytic (about ++), those of Group D still more hemolytic (about +++ ±), and strains included in Groups C and E were the most markedly hemolytic (about ++++).

TABLE III
Precipitin Tests with Control Group of Streptococcus viridans Cultures

<i>Streptococcus viridans</i>			0.2 cc. antiserum for hemolytic streptococci of Groups				
Strain	Extract		A	B	C	D	E*
		<i>cc.</i>					
1	A 148	0.4	—	—	—	—	—
		0.1	—	—	—	—	—
2	A 84	0.4	—	±	—	—	—
		0.1	—	+	—	—	—
3	V 110 A	0.4	—	—	—	—	—
		0.1	—	—	—	—	—
4	A 49	0.4	—	—	—	—	—
		0.1	—	—	—	—	—
5	A 135	0.4	—	—	—	—	—
		0.1	—	—	—	—	—
6	B 39	0.4	—	—	—	—	—
		0.1	—	—	—	—	—
7	W 73	0.4	—	—	—	—	—
		0.1	—	—	—	—	—
8	A 141	0.4	—	—	—	—	—
		0.1	—	—	—	—	—
9	38 D	0.4	—	—	—	—	—
		0.1	—	—	—	±	—
10	K 157	0.4	—	—	—	+	—
		0.1	—	—	—	±	—

* See footnote Table I for cross-reactions of one antiserum for Group E.

When grown on the surface of blood agar plates the colonies of the last two groups (C and E) had a distinctive appearance.

After 16 hours' incubation they were translucent and many had a dewdrop or mucoid appearance. Frequently, two types of colonies were observed, one more mucoid and the other almost pin-point; this type tended to become predominant after prolonged cultivation on artificial media. Animal passage restored the

mucoïd colonies. This was especially true of Strain P 230 of this series ("Strain A" of Dr. Theobald Smith (20) who isolated it from lymphadenitis in a guinea pig). This strain was highly virulent for rabbits, and passage through animals of this species was far more effective in restoring the mucoïd colony form and the accompanying high degree of virulence than passage through other animals. Blood agar cultures of these strains changed markedly in appearance after 2 or 3 days either at room temperature or in the incubator. The areas of hemolysis, which at first surrounded the individual colonies, soon merged until the whole sector on which the culture was plated became hemolyzed. At the same time the colonies became transparent and seemed to undergo lysis to a certain extent, although they did not entirely disappear. It was suggested in a previous paper (16) that this might be due to the presence of a bacteriophage associated with these cultures, especially since the majority of these strains were markedly susceptible to the action of a bacteriophage isolated from sewage (15).

The general question of virulence among the strains of the groups described was not systematically investigated, although incidental observations were made. The strains of Group A, largely of human origin, were usually of low virulence for laboratory animals, although in most instances this could be enhanced for a given species by repeated passage. Some of the strains of Group B were highly virulent for rabbits and mice, for example Strains O 90 and K 158 A; and most of those of Group C were virulent for these animals and for guinea pigs as well. It was, indeed, usually difficult to immunize rabbits with living cultures of members of this last group. No information in regard to virulence is available concerning members of the other groups.

DISCUSSION

The data presented in this paper show that hemolytic streptococci can be differentiated serologically by means of the precipitin reaction into distinct and sharply defined groups which are not disclosed by the agglutination reaction. The test is relatively simple and gives results which are strikingly uniform and consistent. The reagents essential in carrying out the test are heat and hydrochloric acid extracts of the microorganisms and the sera of rabbits immunized with formalinized cultures.

One hundred and four of the 106 strains of hemolytic streptococci in this study were classified into five groups, while each of the two remaining strains was different from all others in the series. Ten strains

of *Streptococcus viridans*, included as controls, did not fall into any of these groups. The strains of hemolytic streptococci were of human, bovine, and other animal origin, and also included several strains from cheese. They were obtained from numerous laboratories, chiefly in this country and Canada, with a few from England and Germany. It is an interesting phenomenon that the animal source was closely correlated with this grouping. Thus, Group A comprised chiefly strains of human origin; Group B, chiefly strains of the high acid-producing, sodium hippurate-hydrolyzing variety isolated from bovine and dairy sources; Group C contained strains from a variety of animal sources and included those strains of bovine origin which attained an intermediate final pH of about 4.8 and did not hydrolyze sodium hippurate; Group D included strains from cheese only; and Group E comprised three strains from certified milk, and may be only a small number representing a larger group. Doubtless, other groups would be found if streptococci from other sources were examined, but this series seems sufficient to establish the principle of specific group differentiation by serological methods. The subdivision of some of these groups into specific types has been accomplished by methods discussed elsewhere, and further work on this subject will be reported in another paper.

A few minor cross-reactions between groups were observed, but they were so slight as not to interfere with the specific identification of the strain in question. The results of the serological method of differentiation were brought into relationship with those based on biochemical and cultural reactions by a comparative study of the tests which, from the rather large number available, had been found most satisfactory by other investigators, together with an additional means of differentiation dependent upon marked susceptibility to streptococcus bacteriophage. The striking correlation between the results of the serological method and those based on biochemical and cultural reactions confirms the classification obtained by the serological method and adds much weight to its validity. Members of the groups differentiated by either method can usually be distinguished by their biochemical characteristics; they are, however, much more easily and specifically identified by the anti-C precipitin test.

The specificity of the precipitin reaction is probably dependent upon

the occurrence in all the groups of chemically related but serologically specific substances. The group-specific substance found in strains of human origin has been identified as carbohydrate in nature. At present, however, no information is available concerning the chemical composition of the determinative substances which differentiate the other groups.³ Although the carbohydrate C, identified in members of Group A, was formerly thought to occur in all hemolytic streptococci, study of strains from different animal sources now indicates that other groups of hemolytic streptococci elaborate similar substances distinct from the C fraction of Group A and characteristic for each specific group. This is the basis of the serological test used in the present classification.

SUMMARY

1. All except two of 106 strains of hemolytic streptococci isolated from man, other animals, milk, and cheese have been classified into five groups, which bear a definite relationship to the sources of the cultures. These broad groups may be subdivided into specific types by methods discussed elsewhere. The specific group classification is made possible by employing two special reagents: (*a*) extracts prepared by treatment of the bacteria with hot hydrochloric acid, and (*b*) serum of animals immunized with formalinized cultures. This differentiation is not detected by the agglutination reaction. The grouping agrees with that described by other investigators on the basis of cultural and biochemical characteristics.

2. The group-specific substance present in strains of Group A has been identified chemically as carbohydrate in nature. The chemical composition of the specific substances upon which the specificity of the other groups depends has not been determined. It seems not unlikely, however, that all of them may belong in the general class of carbohydrates, each being chemically distinct and serologically specific in the individual groups.

³ At the time that proof was read (Feb. 25, 1933), additional data were available which show that the substance which determines the specificity of Group B is also carbohydrate in nature.

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