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Detection by Hemagglutination of Antibodies to Group A and Group E Streptococci by the Use of *O*-Stearoyl Derivatives of Their Cell Wall Carbohydrate-grouping Antigens

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The streptococcal group A and group E cell wall polysaccharide antigens were extracted with trichloroacetic acid from the cell or cell wall and esterified with stearic acid. The stearoyl derivatives contained 5 to 8% (by weight) of the ester. Sheep or human red blood cells were sensitized with the esterified antigens and were shown to agglutinate in the presence of specific rabbit antisera. Sera from (i) children hospitalized with group A streptococcal respiratory disease and (ii) swine possessing group E streptococcal lymphadenitis were shown to possess antibody titers significantly higher than the controls. The use of the two esterified antigens as controls for each other established the specificity of the reaction in each case. The general shape of the antigen-antibody precipitin curves was not changed when the stearoyl antigens were used; however, the quantitative aspects differed markedly. Oligosaccharides which inhibit the normal antigen-antibody precipitin reaction did not inhibit the hemagglutination reaction. The adsorption of antisera with whole streptococcal cells reduced the hemagglutination titer in relation to the quantity of cells employed. Data are given on the (i) optimal concentration of stearoyl antigen for sensitization, (ii) time of adsorption of antigen to red cells, (iii) use of albumin as diluting fluid, and (iv) condition of red cells. Properties of the esterified antigens and the mechanism of the agglutination reaction are discussed. The results indicate that polysaccharide antigens of other bacteria may be esterified and employed in a similar manner.

The recent work of Hammerling and Westphal (4) demonstrated that dextran can be esterified with stearic acid and, in such form, will sensitize red blood cells so that they agglutinate in the presence of antidextran rabbit serum.

The streptococcal group A and group E cell wall carbohydrate antigens were prepared in reasonably pure form by Slade (10) and were shown to be suitable for the preparation of stearoyl derivatives (4). It was considered worthwhile to investigate certain of the characteristics of such modified antigens and their usefulness in the detection of specific group antibodies in human and animal sera. A preliminary report of this work has been presented (H. D. Slade and U. Hammerling, Bacteriol. Proc., p. 60, 1966).

Little information is available as to the antigenicity in man of the streptococcal group A cell wall carbohydrate antigen, or of the other streptococcal carbohydrate antigens, due to the lack of suitable methods for their detection. Double diffusion in agar gel (3), agglutination of cell walls (1), or the hemagglutination of red cells sensitized with carbohydrate-mucopeptide complex from the cell wall (8) lack sensitivity or are cumbersome procedures. Information on the presence of such antibodies would be of value in the serological identification of the infecting organism, the time-course of the infection, and studies on the epidemiology of streptococcal disease.

MATERIALS AND METHODS

Growth of streptococci. The cells were grown in 2-liter quantities in Todd-Hewitt broth plus a glucose-

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salts mixture (5), centrifuged, washed three times with water, and lyophilized.

Extraction of antigen. The group-specific carbohydrate cell wall antigen was extracted from whole cells of group A (strain C203) and group E (strain 5385) by the trichloroacetic acid procedure of Slade (10). The quantity of antigen required at the equivalence point in the antigen-antibody reaction in each case was about 0.8 μg. The conditions of the quantitative precipitin determinations were those previously described (10).

Esterification of antigen. The antigens were converted to the *O*-stearoyl derivatives by the procedure of Hammerling and Westphal (4). The *O*-stearoyl content of the group A preparations used (698A and 698B) varied from 6 to 9% (by weight), and the E preparation (706D) contained 5%. The material was dissolved in water and kept frozen when not in use. After 21 months of storage, such a solution was still satisfactory for use. The ester group content of the antigen was determined by the method of Snyder and Stephens (9).

Sensitization of sheep red blood cells. Citrated cells were stored at 4 C for 10 days before use. The cells were washed five times daily from the citrate solution in 0.85% NaCl and suspended to 1% (v/v). A 1.25-ml amount of 1% cells was mixed with 0.06 ml of 0.85% saline containing 250 μg of group A or 100 μg of group E antigen-stearic acid-ester per ml. It is important to obtain a rapid mixing of cells and antigen. The antigen solution was added in small drops during the mechanical agitation of the red cells. After incubation for 30 min at 37 C with occasional mixing, the sensitized cells were centrifuged, washed three times with saline, and suspended to 0.5% (2.5 ml) in saline.

Adsorption and heating of sera. All sera were held at 56 C for 30 min and then adsorbed with one-tenth volume of packed sheep cells for 15 min at room temperature.

Antibody titration. Doubling dilutions of sera were made in 0.85% saline containing bovine, human, or rabbit albumin in Cooke microtiter trays. Each 0.025 ml of diluted serum was mixed with 0.025 ml of 0.5% sensitized red cells. The agglutination patterns were read after standing overnight in the cold. The lowest dilution showing agglutination was considered the end point.

Precipitin tests were performed by the method of Swift et al. (13). Antisera were prepared in rabbits by the injection of whole streptococcal cells according to the procedure of Slade and Slamp (11) or used as received from the National Communicable Disease Center. These sera had been adsorbed with whole cells of a heterologous serological group before use.

RESULTS

It was of interest initially to determine whether the esterification of the antigen with a carbon chain of 18 carbon atoms, as is present in stearic acid, would mask the specific receptor sites. A positive precipitin test was obtained with as little as 125 μg of either group A or group E stearoyl antigen per ml in the presence of specific rabbit antiserum. These results indicated that, if the stearoyl antigens would indeed adhere to the surface of normal red cells, sufficient receptor sites would probably remain available to produce agglutination. Preliminary tests with rabbit anti-A and anti-E streptococcal sera, and sera from children with active streptococcal infections, showed a sufficiently high agglutination titer to warrant further investigation.

Specificity of the hemagglutination. The specificity of the reaction was determined by the use of rabbit antisera to the other serological groups of streptococci. These sera were tested against sheep cells which had been sensitized with either the A or E stearoyl antigens.

Table 1 shows that both A and E sera reacted in high titer to their respective stearoyl antigens. Sera to the other groups did not possess a significant titer. An exception, however, was the case of the A mutant. This species possesses an atypical cell wall group A antigen which lacks the terminal *N*-acetylglucosamine of the side chain, and will cross-react with anti-A rabbit serum (6). A similar finding is shown in Table 1.

Adsorption of antibodies from serum by whole streptococcal cells. Additional proof of the specificity of an antigen-antibody reaction can usually be obtained from the adsorption of the antibody by the bacterial cell. Lyophilized cells representing various serological groups of streptococci were used in an experiment to adsorb rabbit A and E antisera (Table 2). The antibody levels of the adsorbed sera were then determined. In each case, the hemagglutination procedure demonstrated an almost complete adsorption of homologous antibody, whereas, in the cases of heterologous antibody, a reduction of the titer by one-half was usually obtained.

Table 3 shows the specificity of the hemagglutination reaction using the stearoyl antigen, and illustrates that approximately 15 mg of streptococcal cells were necessary to reduce the titer to the control value. Adsorption of the same serum with group E cells did not cause a significant reduction.

Optimal concentration of stearoyl antigen for sensitization of red cells. A limited series of observations was made as to the optimal quantity of the A stearoyl antigen for sensitization. Table 4 shows that either 15 or 30 μg of an antigen containing 6% ester was sufficient to sensitize 2.5 ml of 0.5% red cells. In the latter case, however, the adsorption of additional antigen doubled the agglutination titer. An increase to 45 or 60 μg of antigen increased the fragility of the red cells, and varying degrees of hemolysis were obtained. In addition, the end point was not definite.

In contrast to the A antigen, only 40% as

TABLE 1. *Anti-A and anti-E hemagglutination titers of streptococcal rabbit antisera*

Antiserum	Red cells sensitized with stearoyl ester of group antigen	
	A	E
Normal	4	4
A	4,096	4
A mutant[a]	512	4
B	2	4
C	0	4
D	0	8
E	0	2,048
F	0	4
G	2	4
H	2	2
K	8	4
L		2
M		2
N	0	2
O	0	2
P	0	4
Q	0	4
S	4	2

[a] Strain K43v was used in preparation of A mutant antiserum.

TABLE 2. *Effect of adsorption of anti-A and anti-E rabbit sera with streptococci from various serological groups on the hemagglutination titer*[a]

Sera adsorbed with cells from streptococcal group	Titer after adsorption, red blood cells sensitized with stearoyl group antigen	
	A	E
Normal serum	4	8
Control (no adsorption)	2,048	2,048
A (T3m)	128	1,024
A mutant (K43)	1,024	
B (10790)	1,024	
C (12033)	1,024	
G (9603)	1,024	
E (5385)	1,024	256
F (H60R)		2,048
H (Hockley)		2,048

[a] An 0.2-ml amount of serum plus 10 mg of streptococcal cells were held at 37 C for 2 hr and refrigerated overnight. An 0.2-ml amount of 0.85% saline was added, mixed, and centrifuged, and the supernatant fluid was tested. The strain of streptococcus used for adsorption is given in parentheses.

much E antigen was required to sensitize the same number of red cells.

Conditions of red cells for use. Citrated sheep cells, between 10 and 30 days after bleeding, were used routinely in this work. Human O (blood bank) cells were also used. The latter always gave higher control titers with human sera. Low serum dilutions with both control and positive sera caused clumping of the O red cells, whereas sheep cells usually formed a smooth deposit which covered the entire well.

TABLE 3. *Effect of adsorption of anti-A streptococcal serum with group A and E streptococcal cells on the hemagglutination titer*

Serum[a]	A cells[b]	Titer	E cells	Titer
	mg		*mg*	
Normal	0	4	0	4
Anti-A	0	2,048	0	2,048
Normal	5	4	5	4
Anti-A	5	1,024	5	2,048
Normal	10	2	10	2
Anti-A	10	256	10	2,048
Normal	15	2	15	2
Anti-A	15	16	15	1,024

[a] Serum (0.2 ml) plus lyophilized cells (in quantities indicated) were used. Serum-cell mixture was held at room temperature for 2 hr with occasional agitation and refrigerated overnight. An 0.2-ml amount of saline solution was added, mixed, and centrifuged, and the supernatant fluid was tested.

[b] Group A cells were of strain N19, type 19; group E cells were strain K129, type 1.

TABLE 4. *Effect of quantity of A-stearoyl antigen on sensitization of red cells*

Serum[a]	Antigen to cells (μg/2.5 ml)[b]	Titer[c]	Titer[d]
Control	15	0	0
Anti-A	15	1,024	1,024
Control	30	0	4
Anti-A	30	2,048	4,096
Control	45	4	8
Anti-A	45	4,096[e]	8,192[e]
Control	60	8	8
Anti-A	60	4,096[e]	8,192[e]

[a] Control was normal rabbit serum.

[b] Antigen solution contained 250 μg of group A stearoyl antigen per ml (ester content, 6%).

[c] Cells were used immediately after sensitization.

[d] Cells were used 24 hr after sensitization.

[e] End point began to trail, and varying degrees of hemolysis occurred in lower dilutions of serum.

It is recommended that each batch of cells be titered against standard rabbit antiserum. On one occasion during the late spring and early summer, the cells received from our supplier did not readily agglutinate after sensitization with the stearoyl antigen.

Effect of time on adsorption of stearoyl antigen by red cells. An adsorption period of 30 min at 37 C was adopted as standard for these investigations; however, tests after 5, 10, 20, and 60 min gave similar results.

Use of rabbit serum, human or bovine albumin. When testing the effect of the three additives on the hemagglutination titers against human and rabbit group A antiserum, 0.2 to 0.5% of normal whole rabbit serum gave significantly higher titers than either bovine or human albumin (Table 5). Normal rabbit serum always gave negative or low control titers.

Considerable differences have been found, however, when testing human sera. Control titers were objectionably high when either bovine albumin or rabbit serum was used in the diluting fluid but were satisfactory when human albumin was used. The results also show that 0.1% human albumin was preferable when testing human anti-group A sera. No tests were made with swine albumin when testing swine serum against red cells sensitized with the E stearoyl antigen.

TABLE 5. *Effect of human, rabbit, and bovine serum albumin on hemagglutination*

Protein additive	Sera tested			
	Normal rabbit	Rabbit anti-group A	Normal human	Human anti-group A
Human[a]				
0.1%	0	256	4	1,024
0.2%	0	256	0	256
0.3%	0	64	4	128
0.4%	0	4	4	128
0.5%	0	8	4	128
Bovine[b]				
0.1%	0	8	4	64
0.2%	0	128	32	256
0.3%	0	128	32	512
0.4%	0	128	32	512
0.5%	0	128	32	128
Rabbit[c]				
0.1%	0	128	32	256
0.2%	0	1,024	32	256
0.3%	0	1,024	32	128
0.4%	0	1,024	32	128
0.5%	0	1,024	32	128

[a] Human albumin fraction V, was from Nutritional Biochemicals Corp., Cleveland, Ohio.

[b] Bovine albumin powder, was from the Armour Laboratories, Inc., Chicago, Ill.

[c] Lyophilized whole rabbit serum.

Quantitative precipitin reactions with stearoyl antigens. The precipitin curves of the A and E stearoyl antigens changed markedly when compared to the unesterified preparations (Fig. 1 and 2). The quantity of antigen required at the equivalence point is four- to sixfold, and the quantity of protein precipitated is one-half to one-third that of the control.

Detection of antibodies to A and E streptococci in human, swine, and rabbit sera. The sera from 40 children hospitalized for upper respiratory disease were tested. These children were bled within 24 hr of their admission to the hospital, or 2 to 3 days after the onset of respiratory infection. Serial bleedings were made during the course of their stay in the hospital. In many cases, group A streptococci, which could be identified as to type, were isolated. In other cases, no type antigen could be identified in the group A organism isolated, and in a few instances no group A isolation was obtained.

A representative group of sera from among those examined is shown in Table 6. Approximately 80% of those from which a group A streptococcus was isolated possessed elevated titers against the group A carbohydrate antigen. Serial serum samples taken at 1-month intervals show that the antibody titer was maintained for at least 4 months. Elevations were also obtained in sera from cases in which the group A streptococcus was not isolated. However, in many

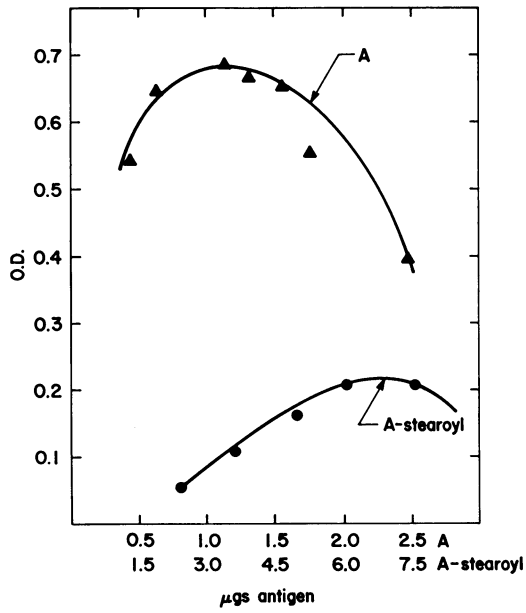

FIG. 1. *Quantitative precipitin curves of esterified and unesterified A antigens against rabbit antiserum. Procedure of Slade (10) was used.*

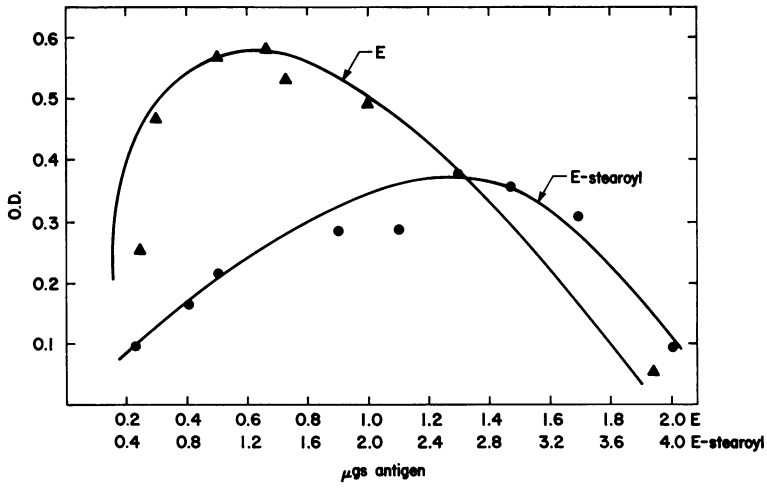

FIG. 2. *Quantitative precipitin curves of esterified and unesterified E antigen against rabbit antiserum. Procedure of Slade (10) was used.*

TABLE 6. *Group A antibody titers in human sera*

Serum no.	Date of bleeding	Titers[a]			Streptococcus isolated	
		AA	ASO	AH	Group	Type
4494	5/4/65	64, 64	100	48	(Non-A)	
	5/19/65	64, 64	100			
	5/25/65	64, 128		48		
	6/29/65	128, 128				
4478	4/23/65	1,024, 512	625	768	A	12
	5/14/65	1,024, 512	833			
	5/28/65	512, 512		384		
	6/28/65	512, 512				
4444	3/29/65	512, 256	125		A	3
	4/15/65	1,024, 1,024	250			
	5/3/65	1,024, 1,024				
	6/7/65	1,024, 1,024				
4513	6/14/65	512, 1,024	166		(Non-A)	
	7/12/65	1,024, 1,024	166			
	7/26/65	512, 512		1,536		
	9/2/65	1,024, 512				
4407	2/4/65	1,024, 1,024	2,500	64	(Non-A)	
	3/18/65	1,024, 1,024	2,500			
	4/8/65	1,024, 1,024				
	5/7/65	1,024, 1,024				
4386	2/9/65	512, 1,024	125		A	3
	4/15/65	1,024, 1,024	333			
	5/3/65	1,024, 1,024				
	6/7/65	1,024, 1,024				

[a] ASO (anti-streptolysin O) and AH (anti-hyaluronidase) titers were determined in the laboratory of Dr. Siegel. AA = anti-group A antibody titer.

instances the elevated anti-streptolysin O or the anti-hyaluronidase titer (or both) indicated the presence of a streptococcal infection. Approximately 200 normal sera were examined. Titers varied from 0 to 128, with a mean of 32.

The association of group E streptococcal infection with an abscess in the cervical lymph nodes of swine has been known since 1937 (7). Sera from both immunized and infected pigs were tested (Table 7). It is apparent that immunized, as well as experimentally infected, animals produce a significantly elevated titer of anti-E agglutinins, and that no significant level of anti-A agglutinins was present.

DISCUSSION

The results presented indicate that stearoyl derivatives of group A and group E streptococcal cell wall carbohydrate antigens adhere to red cell surfaces, and such cells will agglutinate in the presence of specific antibody. It is of interest to estimate the degree of esterification and its relation to the number of combining sites possessed by the antigen. The extraction of group A and group E cell wall carbohydrate antigen from streptococci by trichloroacetic acid (10) yields material, after purification, with a molecular weight of approximately 10,000 (H. D. Slade et al., Bacteriol. Proc., p. 46, 1965). The number of hydroxyl groups available for esterification in such material is unknown and would depend a great deal on the extent of cross-linking between the C_6 units; however, a meaningful calculation can be made.

The E antigen contains glucose-rhamnose in a 1:2 ratio, with serological specificity residing principally in a terminal β-D-glucose (H. D. Slade et al., Bacteriol. Proc., p. 46, 1965). Each terminal glucose would possess four hydroxyl groups, whereas all other C_6 units would contain no more than two, providing no cross-linking existed. An ester content of 5% would require the esterification of only two C_6 units out of a total of 56. In the case of group A antigen, an ester content of 8% would account for the esterification of only three C_6 units. Thus, it is likely that only a small number of the available hydroxyl groups have been esterified. However, a difference can be detected between sheep red cells sensitized with the two antigen esters. Group E cells pack tightly in the centrifuge and do not drain down the side when the tube is inverted. Conversely, group A cells drain readily when the saline washings are decanted. Also, approximately 2.5 times as much A stearoyl antigen (6% ester) is required for sensitization of red cells as with the E preparation (5% ester).

TABLE 7. *Group E hemagglutination titers in sera from immunized and infected swine*[a]

Sera	Red cells sensitized with stearoyl antigen of:	
	Group E	Group A
Normal control	8	4
Immunized	1,024	8
Immunized, experimentally infected, abscess formed	32	8
Immunized, experimentally infected, no abscess formed	256	16
Nonimmunized, abscess formed	2,048	16

[a] Swine were immunized orally with group E modified viable vaccines; sera were drawn 2 weeks after challenge.

The difference in the quantity of antigens (which possess approximately equal ester contents) required to produce optimal sensitization of red cells is of interest. The differences between starch, dextran, and polysaccharides from several gram-negative bacteria have been noted (4). These differences are most likely related to the structure of the polysaccharides, which in turn determines the nature and number of antibody-combining sites.

The successful use of esterified antigens for hemagglutination depends upon effective adsorption of the antigen to the red cell and availability of sufficient combining sites on the antigen after adsorption. It would appear that the optimal percentage concentration of ester in the antigen to achieve these objectives, in the case of the A and E preparations, lies between 5 and 8. A higher degree of esterification would require the use of more antigen in the test in order to provide the proper number of combining sites. This effect has been demonstrated with an A preparation containing 15% ester. The quantity of antigen necessary to avoid hemolysis was not sufficient to provide sensitivity to hemagglutination.

In this study, it has been shown that the stearoyl derivatives of the A and E antigens react with their own antibody (Tables 1–3, 5, 6). One example of a cross-reaction was found (Table 1). Antiserum to a group A mutant cell shows a significant titer to cells sensitized with the stearoyl derivative of the normal antigen. The antigen of the mutant cell exhibited an altered specificity due to the loss of the terminal side chain compound responsible for the main part of its specificity, *N*-acetylglucosamine (6).

The serological specificity of the group E

antigen was shown to be due, in large measure, to β-D-glucose attached to L-rhamnose (H. D. Slade et al., Bacteriol. Proc., p. 46, 1965). Effective inhibition of the precipitin reaction was obtained with D-glucose and a glucose-rhamnose disaccharide isolated from a partial acid hydrolysate of the polysaccharide (H. D. Slade et al., Bacteriol. Proc., p. 46, 1965). It was thus of interest to find that no significant inhibition of the hemagglutination reaction occurred with either glucose or several oligosaccharides isolated from the group E antigen. Also, the group A hemagglutination reaction was not inhibited by *N*-acetylglucosamine. Tanned red blood cells sensitized with cell wall mucopeptide-group A polysaccharide were also not inhibited in hemagglutination by *N*-acetylglucosamine (8). These results indicate that the determinants of the esterified antigen, when fixed to red cells, react only with antibody combining sites which will not react with low-molecular weight substances lacking the appropriate configuration. This might be due to a preferential esterification of such antigen sites owing to their accessibility, and would be expected to result in a quantitative change in the precipitin reaction.

The precipitin curves (Fig. 1 and 2) illustrate that the quantitative aspects of the antigen-antibody combination are affected by esterification. More antigen is required at the equivalence point and less antibody nitrogen is precipitated when compared to the unesterified control. In attempting to reach an explanation, it should be kept in mind that all the antigen molecules may not contain the same quantity of ester, the solubility properties of the esterified antigen-antibody complex are likely to be different from that of the normal complex, and esterification of the antigen may have caused a change in the number of combining sites. The length of the stearic acid chain (18 carbon atoms) very likely exerts a steric hindrance effect on some of the active sites of the antigen.

The available evidence indicates that the lipid moieties of the gram-negative cell wall lipopolysaccharide and the red cell membrane are involved in fixation of one to the other. Hydrophobic bonds may be involved (4). The attachment of the stearoyl group to streptococcal group antigen most likely enables the antigen to adhere to red cell membrane. It has been shown that brain ganglioside and phosphatidyl ethanolamine (12) will adsorb to red cells and prevent the attachment of the *Escherichia coli* cell wall lipopolysaccharide. The lipid components of each may be responsible for their adsorption, and the adsorption may occur in a manner similar to that of the group A and group E stearoyl derivatives. It would be of interest to investigate the latter possibility.

Our knowledge of the chemical composition of streptococcal cell wall carbohydrate antigens is limited. However, the composition of the wall (11) shows that three hexoses and two amino hexoses, in varying combination, most likely account for the chemical composition of the antigens (except those composed of teichoic acids) yet to be studied in the streptococci. The esterification of the group A and group E antigens suggests that other streptococcal carbohydrate antigens, and those from other bacteria which possess the appropriate chemical structure, can be similarly esterified and used for the detection of antibodies. A study of esterification, in relation to chemical structure and its effect on the antigen-antibody combination, would be of interest.

Several dozen sera from children with upper respiratory disease were tested; a limited group is shown in Table 5. Elevated hemagglutination titers were most frequently accompanied by isolation of the group A streptococcus from the throat; however, in some cases the organism could not be isolated. The present procedure may be of value in these cases in the identification of the organism present. Anti-streptolysin O and anti-hyaluronidase, indicators of streptococcal infection, are known to be formed in man in response to the antigen formed by group C and group G as well as group A organisms. An elevated anti-streptolysin O or anti-hyaluronidase serum titer does not distinguish between them. The hemagglutination procedure may help in these cases.

Insufficient numbers of sera have been examined to indicate a correlation between the anti-A hemagglutination titer and the anti-streptolysin O and/or the anti-hyaluronidase titers in human sera. The latter are formed in response to the secretion of extracellular substances by the infecting streptococcus, whereas the anti-A titer arises in response to a constituent of the cell wall of the organism. It would be of interest to study the time-course of the development of these three antibodies. Additional studies are also required to determine the relationship between the onset and time-course of the various types of group A streptococcal infection and the rise in anti-A titer. The stability of the anti-A titer over several months (Table 6) is an asset in this respect.

The detection of group E antibodies in immunized and infected swine is an additional indication of value of the hemagglutination procedure. Several streptococcal groups, other than A, have been encountered in infections of the mouth and upper respiratory tract of man; how-

ever, the relationship of the streptococcus to the infections is not clear. A determination of antibody levels in these cases would be of interest. An indication of a slight but significant anti-E titer in human sera was detected in this study.

In the case of a bacterial genus like *Streptococcus*, which is composed of a number of serologically distinct groups, the proof of the specificity of hemagglutination depends upon the use of at least two distinct streptococcal group antigens. In this study the A antigen has served as a control for the E antigen, and vice versa, and the specificity of the procedure has been demonstrated. A recent report (2), employing the stearoyl derivative of A antigen only, has shown an elevation of antibody titers in human and rabbit sera.

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