A COMPARISON OF THE VALUE OF THE AGGLUTINATION AND PRECIPITIN REACTIONS IN THE SEROLOGICAL TYPING OF GROUP A STREPTOCOCCI

ELMA KRUWMIEDE

From Irvington House, Irvington-on-Hudson, New York

Received for publication January 13, 1943

Two methods, slide agglutination (Griffith) and type-specific precipitin tests (Lancefield), are now being used for routine typing of Group A streptococci. Agglutination as a means of type differentiation was first established on a firm basis by Dochez, et al. (1919). It was not, however, until Griffith (1926, 1935) published his work on slide agglutination that interest in typing hemolytic streptococci became widespread. Previously, macroscopic agglutination tests had been employed in studying streptococci; but the difficulties encountered in obtaining stable, diffuse suspensions had prevented their general use. Griffith's method not only reduced these difficulties but also the time and material required.

While Griffith merely attempted to determine type-specificity by revealing the dominant antigen concerned in the agglutination reaction, Lancefield (1940–41) has made more detailed studies of the antigenic structure of hemolytic streptococci. This investigator has shown that at least two antigenic factors, designated M and T, are concerned in the type-specific reactions of Group A streptococci. The M factor, a type-specific protein substance, is present in matted and mucoid variants. Immunization of rabbits with these variants gives rise to anti-M precipitins and type-specific protective antibodies. While the T factor is apparently the important reagent concerned in the agglutination reaction, many types are agglutinated by the M antibody. Although the presence of two different type-specific factors in a single microorganism suggests the possibility of various antigenic combinations, in most instances M and T correspond as to type.

For the past six years the relationship of Group A hemolytic streptococci to rheumatic fever has been studied at Irvington House, a sanatorium for rheumatic children. During this period an attempt was made to type all Group A streptococci isolated from carriers as well as those associated with upper respiratory infections. Since the majority of observers have relied upon slide agglutination, this method alone was employed at the beginning of this study. Although the sera used for slide agglutination were absorbed to remove all non-specific agglutinins for stock strains of all types, cross reactions were often encountered in testing recently isolated cultures. The precipitin reaction was therefore employed to check the slide agglutination findings. The precipitin reaction has been of value not only in determining the type of many strains which showed negative or equivocal results by slide agglutination reaction, but also in establishing the existence of several previously unidentified types.

1 This work was aided by a grant from the Commonwealth Fund.
Strains of known types for immunizing rabbits were either obtained from Dr. Griffith or had been checked by him.

Preparation of vaccines. 100 ml. of broth warmed to 37°C. was inoculated with 30 ml. of rapidly-growing young culture. After 3 hours incubation the culture was killed by adding sufficient boiling physiological saline to bring the temperature to 56°C. and was then maintained at this temperature in a water bath for 15 minutes. The vaccine was centrifuged and resuspended in 125 ml. of physiological saline.

Immunization of rabbits. Rabbits were immunized over a period of from 8 to 10 weeks with vaccines prepared from young heat-killed cultures. Matt or mucoid variants were used in preparing all vaccines since the primary objective of the immunization of rabbits was the preparation of anti-M precipitating sera. Agglutinating sera were often obtained, not only from rabbits who showed an anti-M precipitin response but also from those in whose sera no type-specific precipitins were demonstrable. Following an initial injection of 1 ml. of vaccine the dose was gradually increased until the rabbits received as much as 8 to 16 ml. in a single injection. The rabbits were injected on three successive days each week for three weeks and then bled four days following the last injection. Thereafter they received injections five days a week on alternate weeks; and test bleedings were taken four days following the last injection of each weekly series. The serum was tested for agglutinins and for anti-M precipitins. When the test bleeding showed a 2 to 3 plus reaction with the homologous M extract, or a slide agglutination titer of 1:80 or higher, large bleedings were taken the following day. All sera were absorbed before use.

Absorption of sera

Sera for slide agglutination. It was usually found that agglutinating sera warranted absorption only if the titer for the homologous type was higher than that for any heterologous type. A strain of type 24 (Griffith, Sylvia Turton) which had lost its type-specificity was used for absorption. Three to four parts of undiluted serum were absorbed with one part of heat-killed organisms for 2 hours at 37°C. In most instances, absorptions were repeated until the sera no longer agglutinated suspensions of type 24. They were then tested against several representative strains of all known types and absorbed with those heterologous strains which were agglutinated. From one to nine absorptions were necessary to remove all cross reactions for stock cultures. In many instances, multiple absorptions with a single type were required. A number of the recently prepared sera were found to be satisfactory after a single absorption with living cultures. Therefore, a few of these sera were absorbed with both living and heat-killed microorganisms. The results suggested that a single absorption with living cultures was as effective as multiple absorptions with heat-killed microorganisms of the same type. Sera for slide agglutination were
diluted 1:5 or 1:10, depending upon the titer of the sera after absorption. Those sera which were absorbed with living cultures were filtered through a Seitz filter.

Sera for precipitin tests. Four parts of serum were mixed with one part of packed heat-killed Group A streptococci of a heterologous type, incubated for 2 hours at 37°C, stored in the ice box overnight, and centrifuged the following day. To determine their specificity, the absorbed sera were tested with homologous M extracts from which the Group A carbohydrate had been removed by alcohol precipitation, with heterologous M extracts, and with Group A carbohydrate. When non-specific precipitins were found following a single absorption, the serum was reabsorbed. Occasionally a serum, which showed strong reactions with a number of heterologous M extracts, failed to react with the homologous M extract after absorption, hence was discarded. Serum from which all non-specific precipitins had been removed were filtered through a Seitz filter; then merthiolate was added in a final concentration of 1:10,000.

It was suggested by Eisman (1940) that a single lot of microorganisms could be employed for several absorptions. After being used, the bacterial sediment was washed twice, resuspended in physiological saline, and heated in a water bath at 100°C, for 1 hour in order to destroy any antibody adhering to the bacterial cell. Although bacteria heated in this manner did not prove wholly satisfactory in absorbing sera for the slide agglutination reaction, it was found that they could be used as often as five times in absorbing sera for the precipitin reaction.

Slide agglutination

Suspensions for slide agglutinations were prepared from cultures grown 18 hours either in meat-infusion neopeptone broth with 0.1 per cent NaHPO₄ or in Griffith's trypsin broth (Pauli and Coburn, 1937). The organisms were packed by centrifugation, the supernatant was poured off, and the bacterial sediment was broken up with a capillary pipette. Drops of the suspension were placed on a slide divided into approximately quarter inch squares with a china marking pencil. The sera* were added and mixed with a 1 mm. 28 gauge platinum loop. The slide was rotated, examined with a hand lens, and the agglutination was recorded. When no agglutination occurred, the suspension was stored at room temperature and retested the following day. In many cases, agglutination of a culture appeared to be inhibited by a capsular substance which could be removed by aging at room temperature (Keogh and Simmons, 1940). When a suspension was granular or tended to agglutinate spontaneously, a number of methods were employed in an attempt to obtain workable suspensions. In some instances, satisfactory results were obtained by short periods of incubation or rapid transplants of the cultures; in others, by growing the cultures in broth containing 10 per cent horse serum or 10 per cent active trypsin (Coburn and O'Connell, 1939).

* I am indebted to Drs. Lancefield and Griffith, and to the Lederle Laboratories for many of these sera.
Precipitin tests

M extracts were prepared from the sediment of cultures grown 18 hours in 500 ml. of broth (Lancefield, 1928). It was found essential to use a highly nutrient well-buffered medium in preparing M extracts for precipitin tests. Since non-type-specific precipitins were removed from the sera, removal of the group-specific polysaccharide from crude extracts by alcohol precipitation was omitted. 0.05 ml. of extract was pipetted into small tubes (5 by 50 mm.) and 0.05 ml. of absorbed type-specific precipitating serum was added. Controls of each serum were made with type-specific extract. The tests were read within 15 minutes and after 2 hours at room temperature, and were considered definitely positive only when the reactions with the extracts being tested were as strong as those with the controls. Confirmatory tests with 0.4 ml. of extract and 0.2 ml. of serum were done when the reactions with the extracts being tested were weaker than those with the controls. Reactions with a new lot of serum were considered unreliable until the sera had been used in testing a large number of recently isolated strains of heterologous types. Sera for types 9, 11, 13, and 27 showed marked cross reactions with heterologous extracts even after absorption and were considered unreliable. Since no strains of type 8 were encountered, the value of type 8 serum was unknown. No satisfactory anti-M serum has been prepared for type 24.

Grouping

Hemolytic streptococci were established as members of Group A (Lancefield, 1933) before attempts were made to type them. Polysaccharide extracts were prepared by extraction with either hydrochloric acid (Lancefield) or with formamide (Fuller, 1938).

TYPE DETERMINATIONS

Attempts were first made to type all Group A strains by the slide agglutination method. M extracts were prepared from strains which showed positive agglutination reactions; and precipitin tests were set up with anti-sera for the corresponding types. Since 1939, whenever a strain could not be typed by this procedure, precipitin tests were done with all types for which sera were available. While several factors may be responsible for the agglutination reaction a single antigen, the M substance, is responsible for the type-specific precipitin reaction which parallels the protective antibody response. Therefore, whenever the results obtained by slide agglutination and precipitin tests differed, type determinations were based upon the precipitin reaction. To date, only one strain of Group A hemolytic streptococcus has been reported in which type determination based upon the agglutination reaction differs from that based upon the

* The broth used in these experiments was a modification of that described by Todd and Hewitt (1932). Neopeptone was used and lean beef was substituted for horse meat. The medium was rendered sterile by filtration through a Berkefeld filter.

* I am indebted to Dr. Lancefield for many of these sera.
precipitin reaction (Lancefield, 1940). In the course of this study two groups of strains which appeared to contain the M substance characteristic of one type and the T substance characteristic of another were encountered. These strains have been designated type 10–12 and type 27 (M485).

Type 10–12

Six strains were isolated which appeared to be type 12 by agglutination and type 10 by precipitin test. At the time of their isolation, no type 12 precipitating serum was available. Subsequently, however, a type 12 precipitating serum was obtained from Lederle Laboratories. This serum was indistinguishable from type 10 by precipitin test but was specific for type 12 by agglutination. This group of strains is being studied by Watson and Lancefield (1940).

Type 27 (M485)

Hemolytic streptococci which were agglutinated by type 27 serum were isolated from 72 children. Sixty-two strains were isolated in the winter of 1939–40 (Kuttner and Krumwiede, 1941) and 10 in the summer and fall of 1940. Slide agglutination reactions with many of these strains were weak or showed cross reactions with types 11 and 28. Since no reliable type 27 precipitating serum was available, rabbits were immunized with a strain M485 isolated from a child with pharyngitis, and with type 27 (Griffith's 780 Tate). Precipitating and agglutinating serum was obtained from the rabbits immunized with strain M485 while agglutinating serum only was obtained by immunization with strain 780 Tate. Reciprocal absorption of the agglutinating sera established strain M485 as type 27 (agg.).5 Sera against strain M485 gave positive precipitin reactions with extracts of all 72 strains isolated but failed to react with extracts of strain 780 Tate. The failure of extracts of this strain of type 27 (agg.) to react with anti-M serum prepared from a heterologous strain of type 27 may be explained in one of two ways. Since strain 780 Tate did not elicit an anti-M response in rabbits, it may have contained little or no M substance. On the other hand, all strains of streptococci belonging to type 27 (agg.) may not have a common M substance. In view of this possibility, these 72 strains have been classified as Type 27 (M485).

Provisional new types

Many strains were encountered which could not be typed by precipitin tests. Slide agglutination reactions with many of these were negative or equivocal, with the sera available. Since positive precipitin tests were considered the criteria for type determination in this study, these strains were not immediately classified. Such unclassified strains which appeared to be of special interest were used to immunize rabbits. The sera were tested for homologous agglutinins and precipitins both before and after absorption; and those strains against which specific anti-M precipitating sera were obtained were considered provisional

5 The qualifying term (agg.) indicates that type determination in this instance was based upon the agglutination reaction only.
new types. To date six provisional types have been identified and designated tentatively as provisional types 32 (formerly C51), 33 (formerly R31), 34, 35, 36 (formerly B35) and 37 (Kuttner and Krumwiede, 1941; Kuttner and Reyersbach, 1943). Specific agglutinating sera have been obtained for only two of these: viz. 32 and 36. The tests to determine the specificity of one of these types are presented in table 1. Dr. A. T. Wilson of The Hospital of the Rockefeller Institute has kindly supplied us with sera for 4 additional provisional types designated as provisional types 38, 39, 44, and 45. After absorption the sera for prov. types 38 and 39 contained type-specific agglutinins. The sources of 102 strains belonging to these provisional types are presented in table 2.

![Table 1](http://jb.asm.org/) on September 23, 2017 by guest

* Provisional new type.

The provisional numbers designating these newly recognized types have been assigned as a matter of expediency after conference by Dr. Ann G. Kuttner, Director of Irvington House, with Drs. H. F. Swift and R. C. Lancefield of The Hospital of the Rockefeller Institute.

Prov. is the abbreviation used for provisional in the remainder of paper.
Results of typing

Of 382 strains tested, 333 (87 percent) were typed by anti-M precipitin tests. A summary of the slide agglutination reactions of these strains is shown in table 3.

### TABLE 2
### Sources of provisional new types

<table>
<thead>
<tr>
<th>Source of provisional new types</th>
<th>Isolated at Irvington House</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Provisional Type</strong></td>
<td><strong>No.</strong></td>
<td><strong>Source</strong></td>
</tr>
<tr>
<td>32 U.R.I.</td>
<td>1</td>
<td>Adm. carrier</td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>Carriers U.R.I.</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>U.R.I.</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Adm. carrier</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Carrier</td>
</tr>
<tr>
<td>36 U.R.I.</td>
<td>1</td>
<td>Adm. U.R.I.</td>
</tr>
<tr>
<td></td>
<td>6</td>
<td>U.R.I. Carriers</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Adm. carrier</td>
</tr>
<tr>
<td>38 U.R.I.</td>
<td>1</td>
<td>Adm. carrier</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Adm. carrier</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Carrier</td>
</tr>
<tr>
<td>39 U.R.I.</td>
<td>1</td>
<td>Adm. carrier</td>
</tr>
<tr>
<td>46 U.R.I.</td>
<td>1</td>
<td>Adm. carrier</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Carrier</td>
</tr>
<tr>
<td>33 Carrier</td>
<td>1</td>
<td>Adm. carrier</td>
</tr>
<tr>
<td></td>
<td>22</td>
<td>Carriers U.R.I.</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Carrier</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Adm. carrier</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>U.R.I.</td>
</tr>
<tr>
<td>34 Carrier</td>
<td>1</td>
<td>Adm. carrier</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Adm. carrier</td>
</tr>
<tr>
<td>35 U.R.I.</td>
<td>2</td>
<td>U.R.I.</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>Carriers U.R.I.</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>U.R.I.</td>
</tr>
<tr>
<td>45 U.R.I.</td>
<td>1</td>
<td>Carrier</td>
</tr>
<tr>
<td>37 Carrier</td>
<td>3</td>
<td>Carriers U.R.I.</td>
</tr>
</tbody>
</table>

* See footnote page 122.
† These types were established at The Hospital of the Rockefeller Institute.

Two hundred and three strains (61 per cent of the typed strains) were correctly typed on the basis of the agglutination reaction; while 20, which reacted with a single agglutinating serum, and 14 of the 74, which reacted with two or more agglutinating sera, would have been incorrectly typed had no precipitin tests...
been done. Thirty-six strains failed to react with any agglutinating sera. Although 297 strains (78 per cent of those typed by the precipitin reaction) could apparently be typed by slide agglutination, an error of at least 12 per cent would have occurred if no precipitin tests had been done.

### Table 3
Comparison of slide agglutination and anti-M precipitin reactions

<table>
<thead>
<tr>
<th></th>
<th>(1)</th>
<th>(2)</th>
<th>(3)</th>
<th>(4)</th>
<th>(5)</th>
<th>(6)</th>
<th>(7)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>SLIDE AGGLUTINATION REACTIONS</strong></td>
<td>Type specific</td>
<td>Non-type specific</td>
<td>Multiple agglutinations</td>
<td>Type specific strongest</td>
<td>Non-type specific equal to or stronger than type specific</td>
<td>Negative</td>
<td></td>
</tr>
<tr>
<td>Types designated by Griffith</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Types</td>
<td>strains</td>
<td>strains</td>
<td>strains</td>
<td>strains</td>
<td>strains</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 1</td>
<td>3 (spon. agg.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 2</td>
<td>9 (types 3, 5, 8)†</td>
<td>2 (types 24, 25)†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 3</td>
<td>11†</td>
<td>6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 4</td>
<td>3 (type 11)†</td>
<td>1</td>
<td>4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 5</td>
<td>43</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 6</td>
<td>1</td>
<td>1 (type 23)†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 7</td>
<td>1 (types 15, 19)</td>
<td>4 (types 15, 19, 23)</td>
<td>5</td>
<td>10</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 8</td>
<td>1 (type 6)</td>
<td>1 (types 15, 17, 19)</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 9</td>
<td>1 (type 4)</td>
<td>2 (types 5, 15, 17)</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 10</td>
<td>3 (type 22)†</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 11</td>
<td>1 (type 23)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 12</td>
<td>4 (type 4)</td>
<td>22 (types 11, 28)</td>
<td>1 (types 11, 28)</td>
<td>72</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 13</td>
<td>1</td>
<td>1 (type 6)</td>
<td>1 (types 15, 17, 19)</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 14</td>
<td>1 (type 23)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 15</td>
<td>14 (types 5, 15)</td>
<td>1 (types 5, 15)</td>
<td>20</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 16</td>
<td>26</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 17</td>
<td>1 (type 23)</td>
<td>1 (types 5, 27)</td>
<td>3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 18</td>
<td>2 (types 4, 34, 36)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 19</td>
<td>15 (type 6)†</td>
<td></td>
<td></td>
<td>12</td>
<td>27</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 20</td>
<td>1 (type 23)†</td>
<td></td>
<td></td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 21</td>
<td>1 (type 4)</td>
<td></td>
<td></td>
<td>3</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Type 22</td>
<td>1 (type 23)</td>
<td></td>
<td></td>
<td>1</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>203</td>
<td>20</td>
<td>60</td>
<td>14</td>
<td>36</td>
<td>333</td>
<td></td>
</tr>
</tbody>
</table>

Spon. agg. = spontaneous agglutination.
* Agglutinated by Type 13 serum.
† Types of sera causing the commonest cross reactions are shown in brackets.
‡ Grown with trypsin.
Strains agglutinated by serum of one type only

Although extracts of 203 of the strains which were agglutinated by serum of one type only reacted with anti-M serum for the same type, extracts of 20 others reacted with anti-M serum of other types (table 3, Column 3). These results suggested that these 20 strains might contain the M substance characteristic of one type and the T substance characteristic of another; further evidence, however, indicated that the agglutination reactions of these strains were non-type-specific. For instance, many strains of prov. type 33 tended to agglutinate with type 8 serum after growth in the presence of active trypsin but failed to show a positive precipitin reaction with type 8 serum. Since the value of the type 8 serum for the precipitin reaction was not known, a type 8 agglutinating serum with a higher titer (positive in a dilution of 1:320) was prepared. This serum failed to agglutinate any of the prov. type 33 strains even in low dilutions. The agglutination reactions with prov. type 33 cultures previously obtained with the stock type 8 agglutinating serum were therefore considered non-specific.

Repeated absorption had weakened the sera for types 14, 18, and 19. Furthermore, it had been impossible to remove all non-specific agglutinins from many of the sera. Agglutination reactions were therefore considered non-specific when a strain, belonging to a type for which the agglutinating serum was weak, was agglutinated only by a serum known to contain non-specific antibodies.

Strains agglutinated by serum of two or more types

Many investigators have noted the tendency of some types to show cross reactions when tested by slide agglutination. Plummer (1941) and Rudd, et al. (1939) had difficulty in distinguishing between types 17 and 23, Neisser (1939) and Coburn and O'Connell (1939) between types 15 and 17, and Keogh and Simmons (1940) between types 15, 17, and 23. During the course of this study, although strains of type 15, isolated during an outbreak of pharyngitis, were agglutinated by type 15 serum only, the majority of strains of types 17 (ppt.), 19 (ppt.), and 23 (ppt.) showed a tendency to agglutinate with sera for types 15, 17, 19, and 23. Since some investigators have been able to differentiate these four types, it seems possible that they contain type-specific agglutinogens as well as agglutinogens common to two or more of these types.

Type 4 (ppt.), 26 (ppt.) and prov. type 46 (ppt.) form a second group of organisms which appear to contain a common agglutinogen (Plummer, 1941; Rantz, 1942). Many strains belonging to these types tended to show cross agglutination reactions with sera for types 4, 24, 26 and the prov. type 46 established by Wilson. Attempts to eliminate these cross reactions from the serum for prov. type 46 resulted in the loss of all agglutinins.

The results of slide agglutination tests with strains belonging to these two categories (types 15, 17, 19 and 23; and types 4, 24 and 26) were difficult to interpret. The agglutination reactions with sera for two or more types were often equally rapid and marked. Repeated slide agglutination tests with different subcultures from a single strain often gave variable results.

* The qualification "ppt." indicates type determinations based upon the precipitin reaction.
Many strains belonging to prov. type 32 showed cross agglutination reactions with serum for types 15 and a few with serum for type 5. As may be seen in table 2, prov. type 32 serum failed to agglutinate type 5 strains and agglutinated type 15 strains only feebly before absorption. Since no prov. type 32 cultures were available at the time the types 5 and 15 sera were absorbed, these sera could not be tested or absorbed with prov. type 32. Subsequently a serum for type 15 has been absorbed with prov. type 32 without reduction of the titer for type 15.

**Negative agglutination**

Of the 36 strains (table 3, column 6) which could be identified by the precipitin reaction but failed to agglutinate with any sera, 25 belong to types for which no agglutinating serum was available and 9 to types for which the agglutinating serum was known to be weak. No attempt has been made to determine whether these 9 cultures represent strains containing the M substance of a known type in conjunction with T substance of an unknown type. The failure of a strain of type 23 (ppt.) to agglutinate may be due to lack of specific agglutinins for type 23 as opposed to agglutinins common to types 15, 17, 19, and 23 in the sera used. A granular culture of type 6 failed to agglutinate after growth in the presence of active trypsin.

**Unclassified strains**

Forty-nine strains could not be identified by the precipitin reaction with any of the anti-M sera available (table 4). Twenty-nine of these strains were agglutinated consistently by sera for one or more types. It is not known whether these strains contain little or no M substance or represent organisms containing an unidentified M substance in combination with a known T substance.

One strain was agglutinated strongly by type 1 serum. The organism formed glossy colonies and probably contained little M substance.

**TABLE 4**

<table>
<thead>
<tr>
<th>AGGLUTINATION REACTION (SEIRA)</th>
<th>NUMBER OF STRAINS</th>
</tr>
</thead>
<tbody>
<tr>
<td>Type 1</td>
<td>1</td>
</tr>
<tr>
<td>Types 4-24-26</td>
<td>5</td>
</tr>
<tr>
<td>Types 11-27-28</td>
<td>6</td>
</tr>
<tr>
<td>Type 12</td>
<td>2</td>
</tr>
<tr>
<td>Type 13</td>
<td>5</td>
</tr>
<tr>
<td>Types 15-17-19-23</td>
<td>2</td>
</tr>
<tr>
<td>Type 25</td>
<td>4</td>
</tr>
<tr>
<td>Prov. type 32</td>
<td>4</td>
</tr>
<tr>
<td>Irregular results</td>
<td>5</td>
</tr>
<tr>
<td>Total</td>
<td>49</td>
</tr>
</tbody>
</table>
Five strains were agglutinated by sera for types 4, 24, and 26. The strength of the reactions with the different sera varied in different tests. The type 26 reaction was encountered least often and was weakest. One of these strains was isolated from a carrier who subsequently developed pharyngitis due to type 4 (ppt.).

Six strains were agglutinated by types 11, 27, and 28. Since these strains failed to react with type 27 (M485) or type 28 anti-M sera, and because reliable type 11 anti-M serum was not available, they could not be identified satisfactorily. One of these strains was isolated from a carrier who subsequently developed pharyngitis due to type 27 (M485). Since both strains were agglutinated by sera for types 11 and 27, they were difficult to differentiate on the basis of the slide agglutination reaction. A type 27 serum, however, prepared with a strain of type 27 (M485) failed to agglutinate the carrier strain.

Two strains (155K and 426S) were agglutinated by type 12 serum. Extracts of these cultures failed to react with antisera for the M substance common to types 10 and 12. Sera from a rabbit immunized with one of these strains showed specific agglutinins but no anti-M precipitins. Slide agglutination tests with dilutions of this serum and two other type 12 sera were done using suspensions of strains 155K, 426S, type 12 (SF42) and heterologous types. Strains 155K and 426S were indistinguishable from type 12. Although no reciprocal absorptions have been done, these strains have been classed as type 12 (agg.). They probably contain the T substance characteristic of type 12, and may either have an M substance distinct from that found in type 10-12 or be essentially glossy forms deficient in M substance.

Since no reliable type 13 precipitating serum for confirmatory tests was available, five strains which were agglutinated by type 13 serum have been tentatively designated type 13 (agg.).

Cross reactions among types 15, 17, 19, and 23 have made these four types difficult to differentiate by the slide agglutination technique. Although 9 strains showing cross agglutination reactions with these sera were identified by precipitin tests, two strains recorded in Table IV could not be specifically identified by either slide agglutination or precipitin tests.

Type 25 serum agglutinated four strains but did not give positive precipitin reactions with them. The serum from rabbits immunized with one of these strains showed a high agglutination titer for the homologous organism and for type 25 but gave no precipitin reactions with extracts of either.

Four strains were agglutinated by prov. type 32 sera. The reactions, however, were so weak they were considered insignificant. The agglutination reactions of five other strains were too variable to identify the strain; finally, fifteen strains failed to agglutinate with any sera.

Carrier strains

The type of all hemolytic streptococci isolated from children, known to carry Group A strains, was checked once a month by the slide agglutination test. Precipitin tests were omitted unless the slide agglutination reaction or a change
in colony form suggested that a child harbored a different type. A number of children carried Group A streptococci upon admission and many became carriers during their stay in the institution. In accord with the findings of other investigators, cultures isolated from carriers, as compared to those isolated from patients with upper respiratory infections, often tended to agglutinate spontaneously or were inagglutinable. In many instances when such strains could not be typed by slide agglutination, satisfactory precipitin reactions were obtained.

DISCUSSION

An attempt was made to type 382 strains of Group A hemolytic streptococci both by the slide agglutination technique (Griffith) and by the anti-M precipitin reaction (Lancefield). Since several antigens may be involved in type-specific agglutination, whereas a single antigen, the M substance, is probably responsible for the precipitin reaction, all type determinations were based upon the precipitin test. Two hundred and thirty-one strains (60 per cent) were shown to be members of the types described by Griffith. Type classification of all unidentified strains which appeared to be of special interest was attempted by the immunization of rabbits with representative strains. The type specificity of six cultures was determined on the basis of the M precipitin reaction, and these strains were classified as provisional new types. Without the use of the precipitin reaction four of these six provisional new types could not have been established, because attempts to prepare satisfactory agglutinating sera proved unsuccessful. One hundred and two strains (27 per cent) were shown to belong to provisional new types established in this laboratory and in the laboratory of The Hospital of the Rockefeller Institute.

A comparison of the typing results obtained by the slide agglutination reaction and by the anti-M precipitin reactions shows that more definite conclusions could be drawn from the latter. Positive precipitin reactions were obtained with 333 strains (87 per cent), all of which could be readily typed on the basis of the anti-M reaction. On the other hand, while positive agglutinations were obtained with 331 of the 382 strains tested, many of the reactions were unreliable or equivocal.

Unreliable agglutination reactions were often due to sera which had been weakened by repeated absorption and to sera from which all non-type-specific agglutinins had not been removed. The later sera led to false results with three groups of strains: recently isolated strains which appeared to contain larger proportions of non-specific antigen than the stock strains, strains belonging to provisional new types, and strains which were tested repeatedly because they appeared to be inagglutinable. Cross reactions with strains belonging to provisional new types were due to the fact that these types had not been established at the time the sera were absorbed. Therefore, these cross reactions were not eliminated. It was found that unless the presence of capsules interfered with agglutination, non-specific reactions were as apt to be encountered as were specific reactions when repeated attempts were made to agglutinate apparently inagglutinable strains. Unreliable results were also encountered when cultures were
grown in broth containing active trypsin. While such growth was of help in obtaining agglutination with some strains, this procedure often induced non-specific reactions. Keogh and Simmons (1940) are also of the opinion that unreliable results may be obtained by this method.

It is generally recognized that certain types show a greater tendency to cross reactions than others, and that reciprocal absorption of sera for these types may remove type-specific as well as non-type-specific agglutinins. Plummer (1941) has suggested that some types are closely related in their agglutination reactions and may therefore be grouped together. Since the typing of Group A streptococci is of value not only in the study of streptococcal epidemics but also in the study of streptococcal immunity, it is advisable to differentiate all types carefully even though some may be closely related. In most instances this may be done on the basis of the M-precipitin reaction which closely parallels the protective antibody response. During the course of this study some children developed infections due to hemolytic streptococci showing agglutination patterns similar to those encountered with strains they had previously carried. In each instance, although the carrier strain could not be identified, precipitin tests showed that it did not contain the M substance present in the strain isolated during infection. Since anti-M precipitins and protective antibody response correlate, differentiating such strains on the basis of the anti-M precipitin reaction is important in studying streptococcal immunity. Moreover, while combining two or more types, such as types 4, 24, 26 and prov. type 46, may not necessarily detract from the value of individual epidemiological studies, it makes comparison of the results of different investigators unsatisfactory. For example, reports from various localities of a number of epidemics due to types 4-24-26 may lead to the assumption that a single type is causing a widespread epidemic, when, in reality, four types (4, 24, 26 and prov. type 46) may be responsible for scattered outbreaks.

Many of the unreliable and equivocal results encountered with slide agglutination reactions are eliminated by use of the type-specific precipitin test. This test not only removes the problems presented by granular and inagglutinable cultures but also makes it possible to differentiate those types which appear to contain closely related agglutinogens. Sera for use in the precipitin test require fewer absorptions and may be more accurately tested for non-type-specific reactions than those used for slide agglutination. Furthermore, correlation of anti-M precipitins and protective antibodies makes the precipitin test of greater value in studying streptococcal epidemiology. The simplicity of absorbing sera for the precipitin reaction, however, does not eliminate the need for testing each new lot of serum against extracts of all known types and of setting up adequate controls.

Although the precipitin test is of greater value than slide agglutination, the former cannot at present completely replace the latter. Glossy strains can seldom be typed by the precipitin reaction since they contain little or no M substance. Although glossy epidemic-inducing strains have not been reported, complete epidemiological studies require the typing of all carrier strains. Furthermore, large quantities of media and sera are required for routine typing by the precipitin reaction, and to date it has been found impossible to prepare reliable
precipitating sera for all types. Many laboratories will, therefore, find it necessary to continue using the slide agglutination reaction until a complete set of precipitating sera can be prepared, and until some method is devised whereby accurate precipitin tests can be done with small quantities of sera. In the meantime, slide agglutination results should be confirmed in some manner. Plummer (1935) suggests the use of macroscopic agglutinin absorption tests. Macroscopic agglutination, however, involves many of the same difficulties encountered in slide agglutination and measures the same antigens. Type-specific-precipitin tests for confirmation of slide agglutination reactions give results comparable to those obtained by use of the precipitin reactions alone and overcome the greatest objection to their use for routine typing, the quantities of sera required. When the precipitin test is used as a confirmatory test, the necessity for setting up each extract against sera of all types is eliminated. Furthermore, during streptococcal epidemics, only a limited number of strains, which appear to be the same type on the basis of the slide agglutination reaction, need be tested. For complete epidemiological studies of Group A streptococcal infections, therefore, the combined use of slide agglutination and anti-M precipitin typing appears at present to be advisable. By combining these two methods of typing, the greatest amount of reliable information can be obtained.

SUMMARY

1. The results obtained in testing 382 strains of Group A hemolytic streptococci by slide agglutination (Griffith) and type-specific M precipitin reactions (Lancefield) were compared. Although the antigen-antibody systems which govern the former are not necessarily identical with those responsible for the M precipitin reaction, there appears to be a high degree of correlation between the two systems.

2. 333 strains (87 per cent) were typed on the basis of the anti-M precipitin reaction.

3. The isolation of 6 provisional new types is reported; and the value of the precipitin reaction in establishing the specificity of these types is stressed.

4. The difficulties encountered in the slide agglutination reaction are discussed with the suggestion that some means of confirming slide agglutination results should be employed.

5. Precipitin reaction with type-specific M substance has been recommended as an accurate means of confirming type determinations based upon slide agglutination reactions.

6. The results of this comparative study indicate that if a simple and economical anti-M precipitin technique were available, the classification of most unknown strains would routinely be more accurately accomplished by the precipitin than by the slide agglutination method.

*Dr. Homer F. Swift, of The Rockefeller Institute for Medical Research, reports (personal communication) that it is possible to prepare sufficient extract from 40 ml. of broth culture to carry out complete anti-M precipitin typing in capillary pipettes.
REFERENCES


EISMAN, P. C. 1940 Personal communication.


LANCEFIELD, R. C. 1940-1941 Specific relationship of cell composition to biological activity of hemolytic streptococci. The Harvey Lectures, 36, 251-290.


WATSON, R. F., AND LANCEFIELD, R. C. 1940 Personal communication.