1941

Rebecca C. Lancefield, 1941

The Rockefeller University

SPECIFIC RELATIONSHIP OF CELL COMPOSITION TO BIOLOGICAL ACTIVITY OF HEMOLYTIC STREPTOCOCCI[1]

REBECCA C. LANCEFIELD

Associate, The Rockefeller Institute for Medical Research

THE early descriptions of streptococci dealt chiefly with their morphology and pathogenicity, from the time when Pasteur (98) saw them as a chain of beads in the blood of a patient dying of puerperal sepsis until Schottmüller (107), in 1903, showed that various streptococci differ in their effect on blood. He distinguished hemolytic streptococci from *Streptococcus viridans,* according to whether the microorganisms hemolyzed or produced a greenish discoloration of the red blood cells around a colony growing on a blood agar plate. Mandelbaum (88), in 1907, described *Streptococcus saprophyticus,* now commonly called the indifferent streptococcus, which did not produce any visible effect on red blood cells. These observations have been the basis for the main differentiation of streptococci for almost forty years. In 1919, Brown (11, 119) defined these three varieties more exactly and designated them as *alpha,* indicating the slightly hemolytic, methemoglobin producing strains; *beta,* those that were markedly hemolytic; and *gamma,* those without visible effect on blood.

Thus, the so-called streptolysin (6) is the cellular component of hemolytic streptococci that was first useful for classification. Opinions of the earlier investigators varied with regard to the filtrability and antigenicity of this substance, but all were unanimous as to its lability. McLeod (91) agreed with some of these investigators in concluding that streptolysin was not antigenic. He established as a criterion for the production of a soluble hemolysin, the lysis of washed red blood cells by broth cultures or their filtrates. This led to some confusion in the case of those streptococci which hemolyzed whole sections of blood agar plates,

[1] Lecture delivered May 15, 1941.

but failed to yield a "soluble hemolysin" when tested by McLeod's method (21, 129, 135). Neill and Mallory (95) found that, like the lysins of other microorganisms which they had studied, streptolysin was subject to reversible oxidation and reduction.

Todd, beginning in 1928 (128), reinvestigated this whole question and, in 1938 (130–132), offered a more complete explanation of the peculiarities of the lytic activities of hemolytic streptococci. Briefly, he found two streptolysins, streptolysin O and streptolysin S, with radically different properties. Mixtures containing different proportions of the two explained the variable results of earlier work. Streptolysin O, found in serum-free broth culture filtrates, is subject to reversible oxidation and reduction. It is antigenic and combines with antibody either in the oxidized or reduced state, but is hemolytic only when reduced. Antibodies to this streptolysin are formed in response to streptococcal infections, and serve, therefore, in the study of human diseases, as a useful diagnostic index of previous streptococcal infection. Smythe and Harris (120) concluded from chemical studies of partially purified preparations that streptolysin O is probably protein in nature and that it contains rather large amounts of sulphur, part of which exists as an $-SH \rightleftarrows -S-S-$ oxidation-reduction system. If the –SH groups are removed by oxidation or other means, the substance becomes hemolytically inactive.

Todd (131) also investigated the hemotoxin described originally by Weld (138, 139) and distinguished a second highly toxic substance, streptolysin S, in filtrates of serum broth cultures and in serum extracts of streptococci prepared according to Weld's technique. These preparations contain much more streptolysin S than streptolysin O, but all preparations also contain some streptolysin O. Todd showed that streptolysin S probably represents the most active principle of the hemotoxin described by Weld. In contrast to the oxygen-labile streptolysin O, streptolysin S is not apparently affected by oxidation or reduction, but is exceedingly labile to acid, and so sensitive to heat that

it is irreversibly inactivated by storage even at ordinary ice-box temperatures, and can be preserved only at −73° C. Weld showed that extracts containing this substance are highly toxic for mice; and both Weld and Hare (47) studied the pathological effects in animals, but were unable to correlate the lethal and hemolytic titers of extracts. Todd (130), however, explained this discrepancy when he found both streptolysin O and streptolysin S in these extracts, and showed that these substances produced distinct pathological effects on mice (3). Todd found also that antibodies directed against streptolysin S are formed in experimental animals only in response to immunization with living streptococci. The antibody titers in immunized animals, and also in man following known streptococcal infections, are never very high but show, nevertheless, a significant increase. In rheumatic fever patients, Todd, Coburn and Hill (133) concluded, from statistical analysis of the results of serological studies, that the antistreptolysin S titers of rheumatic children decreased during acute attacks and increased during recovery, in contrast to the steady rise in titer observed in non-rheumatic children in response to streptococcal infections or in rheumatic children who escaped recrudescences following streptococcal infections. The low antistreptolysin S titers are also contrasted with the generally high level of antistreptolysin O during the acute phase of rheumatic fever.

Although hemolytic activities were the foundation of the first successful classification of streptococci, the results of the action of the carbohydrate-splitting enzymes of these microorganisms were often employed in early studies for purposes of classification. Many investigators sought orderly arrangements of streptococci by studying their fermentation reactions, by investigating the limiting pH values for growth and viability, and by determining other biochemical and cultural characteristics. Although considerable progress was possible by these methods, there was always overlapping among the classes differentiated and the results were often difficult or impossible to interpret.

Serological classification of hemolytic streptococci was also

attempted early, using the agglutination reaction and the technique of agglutinin absorption. There are, however, many technical difficulties in streptococcus agglutination reactions and, by the beginning of the last war, opinion was divided as to the value and meaning of these results, either for the determination of serologically distinct types or for the differentiation of strains from human and animal sources (37). It was, however, finally established, from study of epidemic strains in Army camps in 1918, that specific serological types existed among hemolytic streptococci pathogenic for man (25).

GROUP PHENOMENA

Several years later, Hitchcock (59) found that, irrespective of the type-relationships, hemolytic streptococci isolated from a wide variety of human diseases all possessed in common a serologically active polysaccharide. This, at the time, seemed confusing because Heidelberger and Avery (54) had just shown that bacterial polysaccharides were responsible for type-specificity of pneumococci. The demonstration was so clear for pneumococci,

TABLE I

Most Characteristic Source

Group A	Man
" B	Bovine mastitis
" C	Most streptococcal animal diseases
" D	Originally identified as cheese strains; also enterococci and other human saprophytes
" E	Normal milk
" F	"Minute"
" G	Large and "minute" — Human nasopharynx usually non-pathogenic
" H	
" K	
" L, " M	Chiefly dogs, recently identified by Fry*

* Unpublished data; personal communication.

that the same principles were thought to apply to other bacteria. For *Streptococcus viridans* (60, 71), this proved true, but hemolytic streptococci offered the first exception to this rule.

The polysaccharide characteristic of hemolytic streptococci comprising a large number of strains pathogenic for man, now designated as Group A, reacted specifically with the serum of animals immunized with any other members of this group. However, a series of strains isolated from lower animals failed to give this reaction, and it was thought, therefore, that other groups of streptococci might also be characterized by carbohydrates specific for each group. This, indeed, proved true, and several additional groups have been identified as shown in Table I, in which eleven groups are designated with their most characteristic sources. Group A is composed of strains usually pathogenic for man; Group B, of those from bovine mastitis; Group C is found in a great many streptococcal diseases of lower animals; Group D, originally identified in strains isolated from cheese, also includes the so-called enterococci and other human saprophytes; Group E is found in normal milk; Groups F to K occur in the human nasopharynx, usually not associated with disease; and Groups L and M have been recently designated by Fry, and include streptococci found so far chiefly in diseases of dogs.

Members of most of the known groups have, however, been obtained in cultures from various parts of the human body. In Table II are listed the groups, members of which have been isolated from man and from other animals. The chief pathogenic streptococci of human, bovine or equine origin are fairly well-known: Group A, in man, Groups B and C in cattle, Group C in horses. Among guinea pigs also, Group C streptococci are known to be the chief pathogens, as well as among most other domestic and laboratory animals, with the possible exceptions of the monkey, the mouse, and the dog. The second column shows the usual distribution of serological groups of hemolytic streptococci in respect to disease in different animals. Occasionally, however, members of groups not usually pathogenic for a species are isolated from undoubted infections, as shown in the third

TABLE II

Pathogenicity of Serological Groups of Hemolytic Streptococci

Animal source	Chief pathogenic groups	Occasionally pathogenic groups	Groups found but apparently non-pathogenic	References
Man	A	B, C, D, F, G, H,	K, L	15, 18, 32, 44, 46, 48, 51, 52, 63, 77, 82, 105, 106, 111
Cattle	B, C	A, G	D, E, H, L	28, 29, 44, 45, 85, 92, 94, 97, 100, 121, 123, 124
Horse	C			5, 26, 27, 28, 29, 97
Monkey		A, G	C	10, 82, 105, 110
Dog	G, L, M		C	53, 82, 101
Chicken	C	A ?		28, 29, 100
Swine		E,* L	G	
Goat				
Sheep				
Fox		M		
Ferret				
Rabbit		A, B		77
Guinea Pig				
Mouse		A, B, C		86

* Strains received from Dr. Stafseth.

column; still others have been found which have never been isolated from disease (Column 4). More work is necessary before closer correlations can be established between serological groups and species susceptibility.

Fatal Human Infections. Investigation of the streptococcal carrier rate in any community is aided by the possibility of determining easily and accurately whether given strains are likely to be pathogenic for man, *i.e.,* those belonging to Group A. It is

of interest to note the distribution of groups which, although they are not usually associated with human disease, may occasionally give rise to infection in man, even sometimes proving fatal. The records found in the literature of thirty-eight fatal human infections due to streptococci of groups other than A are shown in Table III. Compared with the incidence of fatal Group

TABLE III

Fatal Human Infections: Groups B to K

Group	Diagnosis	Number	Remarks	References
B	Puerperal Sepsis	14	Dr. J. H. Brown also reported 45 strains isolated from 2,000 autopsies. He considered few of etiological significance.	13, 20, 36, 56, 105, 106, 117
	Endocarditis	2		
	Terminal pyelonephritis	1		
	Terminal diabetes	1		
	Meningitis	1		
	Pneumonia	1		
		20		
D	Endocarditis	2	Non-hemolytic	112
Probably D	Ludwig's angina	1	Hemolytic	31, 89, 96, 105
	Endocarditis	1	Hemolytic	
	Endocarditis	6	Non-hemolytic (numerous others in literature)	
		8		
F	Pansinusitis	1		8
	Pneumonia with empyema	1	Etiological relationship uncertain	
G	Puerperal Sepsis	4	(with *S. aureus* in 1 case)	19, 90, 105, 117
	Agranulocytosis	1		
	Endocarditis	1		
		6		

A infections, these are relatively few; and usually the streptococci in these cases have been opportunistic or terminal invaders,

as in puerperal sepsis, endocarditis, pyelonephritis, and agranulocytosis. There have also been a few cases of primary infection. Seven cultures from twenty fatal cases due to Group B have been identified in our laboratory, and the remaining thirteen have been reported elsewhere, in the literature. In addition to these, Dr. J. H. Brown isolated from 2,000 autopsies at the Johns Hopkins Hospital, forty-five Group B strains, a few of which might possibly have been the primary agent, although most of them he considered of no etiological significance. No record has been found of a fatal human infection due to Group C, although relatively mild puerperal infections and infections of the throat and urinary tract do sometimes occur. Group C has also been reported as isolated from erysipelas, but its etiological importance here would apear questionable (63). The fatal infections in Group D are interesting because they were apparently all due to the so-called enterococcus and were caused either by hemolytic or by non-hemolytic strains. A few deaths due to Groups F and G are also recorded. No records have been found of fatal human infections caused by members of any of the other known groups.

Non-hemolytic Members of the Various Serological Groups. The correlation between hemolytic capacity and serological grouping is less strict than at first supposed. The serological groups described were originally confined to hemolytic streptococci, but it soon became evident that non-hemolytic strains with apparently identical serological characters were common in Group B. About half of the bovine strains studied by Stableforth (121) were non-hemolytic, although there was no detectable immunological difference between the hemolytic and non-hemolytic members of this group. He suggested that the production of hemolysin might be a variable function. One such variation, indeed, occurred in our own experience (79). The parent strain was of known serological type and was highly virulent for mice. Consequently, it was possible to show by immunological studies that the variant, while maintaining its virulence and antigenic properties, lost only its capacity to hemolyze red blood cells and form pigment; and these properties have never reappeared.

As serological investigations of streptococci have been extended, non-hemolytic strains have been found with more or less frequency among most of the known groups. Completely non-hemolytic members of Group A are rare. Todd (127) studied the transformation of hemolytic Group A strains into methemoglobin producing variants by passage through mice, and Fry (35) noted green-producing variants which were of the same serological type as hemolytic strains isolated from the same patient. These strains were normally hemolytic, if grown anaerobically.

Alpha, or methemoglobin producing, streptococci from bovine mastitis were found by Little (85) to be members of Group C. These non-hemolytic strains constitute a group already well-recognized by the physiological studies of S. J. Edwards (30). Culturally similar, Group C streptococci have also been isolated very recently in England as the etiological agent in several outbreaks of joint-ill in lambs (7) and all belong to a single serological type.

In Group D, non-hemoyltic varieties have been well-studied by Sherman and his associates (111, 112, 116) and by Graham and Bartley (39, 61). The non-hemolytic species, *S. fecalis* and *S. liquefaciens,* not only react as members of Group D, but certain strains belong to the same serological types as hemolytic members of Group D classified biochemically as *S. zymogenes.* These authors are agreed on the close antigenic relationship of hemolytic and non-hemolytic strains of Group D.

Several investigators have found that Group D streptococci vary in their ability to hemolyze red cells from different species of animals. Thus, some strains are non-hemolytic on sheep or goat blood agar, but actively hemolyze red cells from the rabbit and from several other animal species (67). The explanation of this phenomenon is not known.

In other groups, certain non-hemolytic or doubtfully hemolytic strains are sometimes encountered, particularly in Groups H and K.

One serological group, composed entirely of non-hemolytic streptococci, *S. lactis,* has been described by Sherman and his

associates (114). The immunological reactions of *S. lactis* separate it clearly from *S. fecalis* (belonging to Group D), which is in agreement with other biological reactions of these streptococci previously studied by Sherman (115).

The finding of close serological relationship between hemolytic and non-hemolytic streptococci in no way invalidates the usefulness of blood agar as a differential medium in studying these bacteria. This is especially true in Group A where few, if any, frankly non-hemolytic strains are known. The knowledge that both non-hemolytic and actively hemolytic streptococci may belong to the same serological groups directs attention to the fact that they have other fundamental physiological characters in common, which were in some cases overlooked previously on account of the special emphasis placed on the single property of elaborating extracellular hemolysins.

As an illustration, one might cite the recognized insusceptibility of Group D hemolytic streptococci to the action of sulfonamides. With this knowledge, one would question the value of sulfonamide therapy of patients infected with non-hemolytic strains of Group D, members of which are occasionally isolated from cases of subacute bacterial endocarditis or urinary tract infections. In pyelonephritis induced by non-hemolytic Group B strains, on the contrary, sulfonamide therapy would be indicated. Without recognizing the essential relationship of these non-hemolytic strains to the serological Groups B and D, respectively, the clinician might easily mistake both for *S. viridans* and apply the same treatment to both.

Drug Fast Strains. The failure of Group D strains to respond to sulfonamide therapy (9, 34) raises the question of how many Group A strains may also have a natural or acquired resistance to these drugs. Apparently, increasing numbers of resistant Group A strains are being encountered. One wonders, therefore, whether in addition to these naturally drug resistant strains, other Group A streptococci may not acquire fastness to the drug and thus lead to a wide dissemination of strains resistant to these chemotherapeutic agents.

Characteristics of Group Polysaccharides. Although the carbohydrates characteristic of each of the eleven serological groups are convenient tags for identification, the function of these so-called C substances in the streptococcal cell is not understood. There is no group agglutination corresponding to the group precipitin reaction, and the variations in non-type-specific agglutination do not appear to be correlated with the amount of group-specific C antibody present in the serum.

The group-specific carbohydrate, however, in the streptococcal cell seems remarkably constant in amount and apparently does not vary with changes in virulence and in other functions. Since washing the streptococci yields only traces of the group-specific polysaccharide and since cellular disruption is required to obtain it in any quantity, it seems likely that this substance is not present as a capsule but rather forms an integral part of the bacterial cell.

The group-specific carbohydrate of Group A organisms has been studied more than the polysaccharides characteristic of other groups. It is not toxic for animals, and is antigenic only when still in combination with the cellular protein (55). On hydrolysis, this substance yields reducing sugars and according to unpublished results of Sherp (quoted by Heidelberger and Kendall (55)) contains an amino sugar. The precipitin test with this polysaccharide provides a simple method for group identification and differentiation of potential pathogens for a given animal species, but it is obvious that associated biochemical and cultural functions are important in the metabolism and physiology of these bacteria.

EXTRACELLULAR SUBSTANCES ELABORATED BY STREPTOCOCCI

Although the separation of substances elaborated by microorganisms into extracellular and intracellular components is a somewhat artificial distinction, it is useful in contrasting the toxic constituents due to active growth of the streptococcus with those found only after disintegration of the bacterial cells.

Streptolysins. We have already considered the extracellular

toxin which is responsible for the hemolytic properties of streptococci, namely, the streptolysin, or, rather, the two streptolysins, which Todd has shown are characteristic of Group A. Todd (129) studied also the streptolysins produced by strains representative of other groups and found the oxygen-labile streptolysin O in culture filtrates of certain Group C and Group G strains (Table IV), as well as in those of all Group A strains, but the oxygen-stable streptolysin S is quite specific for Group A. Other strep-

TABLE IV

Streptolysins of Group A and Other Groups

Serological group	Streptolysins characteristic of Group A		Other streptolysins
	Streptolysin O	Streptolysin S	
A	Present	Present	.....
C (Matt colony)	Present	.....	Present but little studied
G (Matt colony)	Present	.....	
All other groups	.....	.....	

tolysins, not yet identified either as to number or kind, are obviously present in other serological groups of hemolytic streptococci, as indicated in the last column of Table IV. Although serological identification of these streptolysins has not yet proved feasible, because antibodies have not been obtained for them, Todd has shown that several may be distinguished by their striking differences in susceptibility to the influence of oxygen, of heat and of acid (Table V). Further study may result in the discovery of methods suitable for antibody production specifically directed against these other streptolysins.

Erythrogenic Toxins. In a few laboratories, the capacity of members of the various serological groups to produce erythrogenic toxins has been determined. These toxins are responsible for the rash in scarlet fever and are formed by most Group A

TABLE V

*Characteristics of Streptolysins of Different Groups**

Serological group	Effect on streptolysins of			Streptolysin neutralized by
	Oxygen	Heat	Acid	
A, Streptolysin O	Reversibly inactivated	Destroyed	–	Antistreptolysin O
A, Streptolysin S	–	Destroyed	Destroyed	Antistreptolysin S
B	–	Moderately sensitive	Moderately sensitive	–
C, (''Human'') Streptolysin O	Reversibly inactivated	Destroyed	–	Antistreptolysin O
C, (''Animal'')	–	Destroyed	Slightly sensitive	–
D	–	–	Destroyed	–
E	–	Destroyed	–	–
G, (large colony) Streptolysin O	Reversibly inactivated	Destroyed	–	Antistreptolysin O

* Compiled from Todd's data (129, 132).

– indicates unaffected.

Colony

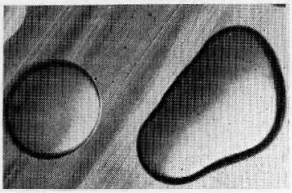
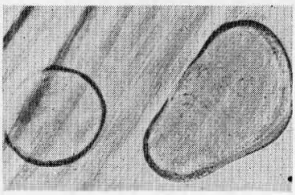
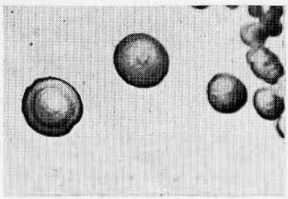
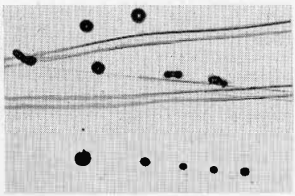
Group C	
"Animal" strains	"Human" strains (Also some animal strains)
 Mucoid	
 After drying: Matt	 Matt
 Small smooth	

FIG. 1.

Forms

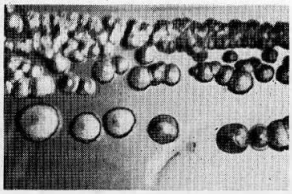
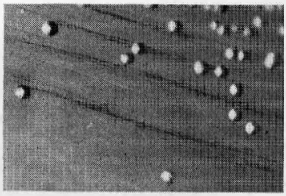
Group G	
Human and animal strains	Human strains
 Matt	 "Minute"

FIG. 1.

strains, but not usually by members of other groups (17, 22, 23, 68, 105).

Fibrinolysin. Another extracellular component of hemolytic streptococci is the fibrinolysin discovered by Tillett and Garner (125). This substance is capable of dissolving the clot of normal human blood, and appears to be enzymatic in nature; but the products of lysis differ from those obtained when fibrin is digested by trypsin. Among the many investigations stimulated by the discovery of fibrinolysin are those relating this phenomenon to particular groups of hemolytic streptococci. The serological groups which form fibrinolysin are the following: Group A, almost without exception; and the large matt colony Group C and Group G strains.

In order to show other relationships of the streptococci of different groups which produce fibrinolysin it may be of interest at this point to discuss the different forms of Group C and Group G strains. Some members of these two groups are linked together by several common characteristics; others from widely divergent sources have little in common except their respective group-specific polysaccharides. In each group, several decidedly different colony forms occur. In Group C, some of the strains widely distributed among lower animals form large mucoid colonies, associated with capsules (Fig. 1), or, if the mucoid substance is absent, they form small smooth colonies. On drying, the colony surface changes from mucoid to matt.

Other Group C strains form large matt colonies which characterize the strains of human, monkey and canine origin and also some of the strains causing equine diseases other than strangles. They are also characteristic of the non-hemolytic Group C strains from bovine mastitis and joint-ill of lambs.

Streptococci of Group G form either minute or large colonies. The former, described by Long and Bliss (87), is a pin point colony with a large area of hemolysis; the latter is a large matt colony indistinguishable morphologically from the matt colonies of Group C, and incidentally of Group A (Fig. 2).

Group C and Group G strains which form matt colonies are

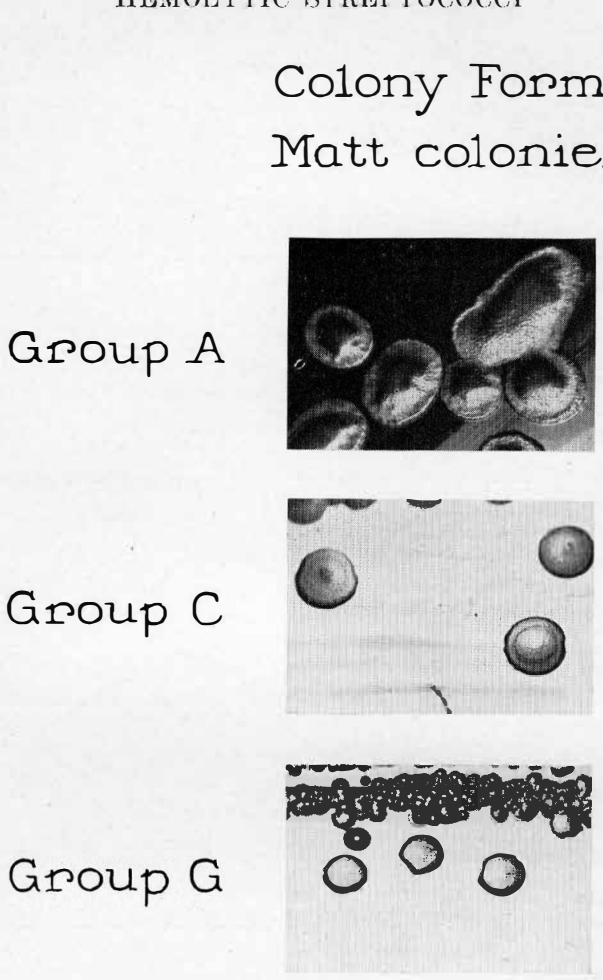


FIG. 2.

also similar in their fermentation reactions with trehalose and sorbitol (Table VI). Both produce the extracellular substances, fibrinolysin and streptolysin O. In these and in other respects, they also resemble Group A to such an extent that they can only be distinguished serologically. They show their close relationship to each other in that they possess a common protein antigen, but this is not encountered in Group A strains. This antigen some-

TABLE VI

Distinguishing Characteristics of Groups C and G

	Group C		Group G	
	"Animal"*	"Human"†	Large Colony	"Minute" (Type 1)
Colony form	Mucoid or small smooth	Large matt	Large matt	Minute, smooth
Hemolytic zone	Wide	Moderate	Moderate	Wide
Capsule	+ (mucoid) − (small)	−	−	−
Fermentation: Trehalose	−	+	+	+
Sorbitol	+	−	−	−
Fibrinolysin	−	+	+	−
Streptolysin O (Group A)	−	+	+	−
Common protein antigen	−	+	+	−
Animal distribution:				
Man	−	+	+	+
Monkey	−	+	+	
Horse	+	+		
Cow	+	+	+	
Sheep	+	+		
Dog	+		+	
Most other lower animals	+			

* Type A of Ogura and Edwards, 97, 26, 27. † Types B1 and B2 of Ogura and Edwards.

times gives rise to confusing cross reactions between certain members of Groups C and G. The cross relationship, however, is not connected with the group-specific polysaccharides of these two groups since their respective polysaccharides when free of this protein are serologically distinct.

Antisera may be prepared free of cross reactions by absorption with appropriate matt-colony Group C or Group G strains or by immunizing rabbits with strains which do not contain this particular protein antigen. Examples of the latter are strains of guinea pig origin in Group C and "minute" colony strains in Group G, but certain technical difficulties sometimes interfere with their use as antigens for group antisera.

Hyaluronic Acid. The last extracellular product of hemolytic streptococci to be considered is the capsular substance, hyaluronic acid, formed by mucoid strains of Groups A and C. Kendall, Heidelberger and Dawson (64) isolated this substance from mucoid Group A hemolytic streptococci and identified it as an acid polysaccharide of high molecular weight composed of acetylglucosamine and glucuronic acid in equimolar concentrations. It functions neither as an antigen nor as a hapten, possibly because it is also normally present in the tissues of animals used for immunization. Sources other than bacterial from which hyaluronic acid has been isolated include bovine vitreous humor, fowl sarcoma, pig skin, Wharton's jelly of human umbilical cord, synovial fluid and the pleural fluid of a patient with mesothelioma.

Seastone (109) studied a similar non-antigenic mucoid polysaccharide from Group C hemolytic streptococci of animal origin. While it has been suggested (86) that the mucoid capsular substance of hemolytic streptococci is related to their virulence, this has not been established in the case of Group A. Seastone, on the other hand, has demonstrated that the variants of Group C which were highly virulent for guinea pigs produced much larger capsules and much more polysaccharide than did strains of low virulence. The different relationship to virulence of this capsular substance in Groups A and C respectively, has been clearly

demonstrated by Hirst (57) who showed that the bacterial cells of both groups were decapsulated *in vivo,* as well as *in vitro,* by leech extract. The decapsulation apparently protected both mice and guinea pigs from fatal infection with Group C streptococci, but was ineffective in mice inoculated with virulent Group A strains. This suggests that substances other than the mucoid polysaccharide are related to virulence of organisms of the latter group. Further evidence indicates that in Group A, the type-specific protein, M, is associated with virulence, and that immune serum containing antibodies specifically directed against this substance protects mice against infection with virulent strains of homologous type.

TYPE RELATIONSHIPS

So far, this discussion has dealt primarily with the relationship between the various cellular constituents and the corresponding biological activities of streptococci in terms of their serological grouping. Let us now examine the specific types so far differentiated within these groups to see what definite activities can be associated with them or with their determinative antigenic components.

Serological Types in Group A. In 1919 (25), we established the principle that type differentiation of epidemic strains could be effected by serological methods. Subsequent studies on the type-specific substances have definitely supported this conception.

In 1926 (40), Griffith attempted by slide agglutination to eliminate one of the great difficulties inherent in all agglutination techniques with streptococci, namely, the difficulty of obtaining stable suspensions. His method also reduced the time and material required for tests. He first established four types, and in 1927, determined that seventy per cent of 222 scarlatinal strains fell into these four recognized types (41). Subsequently, he continued his serological classification and in 1934, published epidemiological studies in which 27 types of hemolytic streptococci were defined (43). Twenty-three of these types were later found to be members of Group A, while the other four types fell into other serological groups: Types 7, 20 and 21 into Group

C, and Type 16 into Group G. Griffith's type classification of Group A hemolytic streptococci has been universally adopted.

Although most of the scarlatinal strains of hemolytic streptococci are included within these types, Griffith and other investigators have found that not all strains isolated from streptococcal infections or from carriers fall into these recognized types. Indeed, Griffith has recently added three more; and other investigators have also described still other types, some of which appear to be more prevalent during certain years and in certain localities.

Griffith pointed out that the methods employed by him, *i.e.*, direct agglutination combined with agglutinin absorption, revealed the dominant antigen which determines type specificity, but he did not attempt to analyze more closely the whole antigenic structure. Investigation of this latter problem has been one of the chief interests in our laboratory; and studies in this direction have brought to light the fact that at least two type-specific antigenic components are concerned with the type-specific reactions of Group A hemolytic streptococci. These components have been designated M and T (81).

Relation of the Type-specific Protein, M, to Colony Form. The type-specific protein, M, is the antigen primarily associated with virulence; and the antibodies to which it gives rise are primarily concerned with the specific protective action of immune sera. It is present only in those variants which form mucoid or matt colonies (Fig. 3). Mucoid, matt and glossy variants are the forms ordinarily observed (126), but Dawson (24) has also obtained a more degraded "rough" form, corresponding to the rough variants in a generalized scheme for all bacteria. The mucoid variant possesses the same antigens as the matt variants and, in addition, produces the serologically inactive capsular substance, hyaluronic acid (64). The matt form may be either virulent or non-virulent, but in either state, matt variants contain approximately equal amounts of the type-specific M substance (83, 134). The glossy forms are always avirulent and contain little or no M substance. They sometimes form mucoid colonies. Immunization of animals with virulent or avirulent

matt variants induces the formation of anti-M precipitins and protective antibodies. Immunization of animals with the glossy

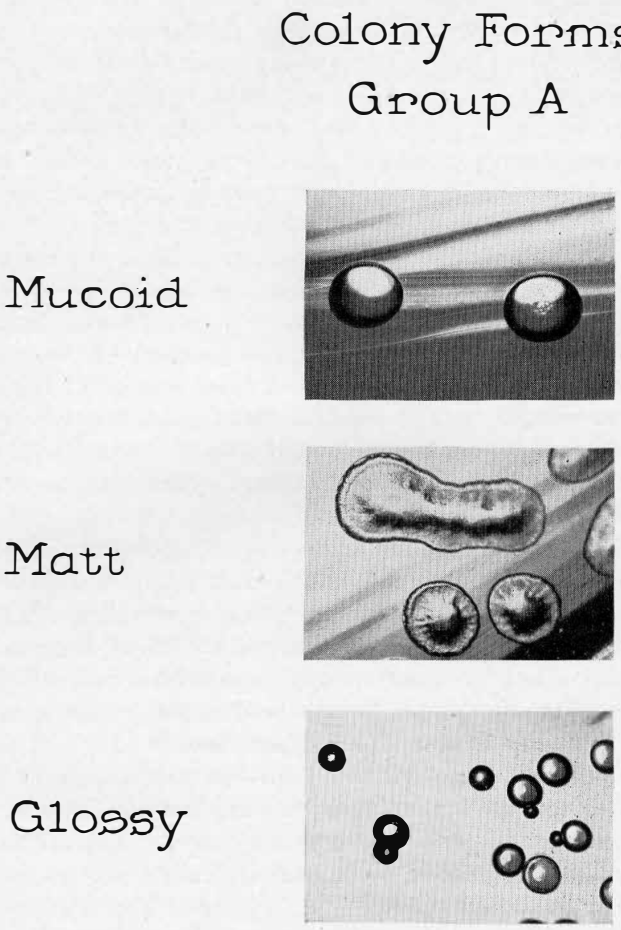


FIG. 3.

form fails to induce either protective antibodies or anti-M precipitins.

Relation of the M Substance to Type-specific Reactions. In its relation to the pathogenic properties of the streptococcus, the M

substance appears to function in a manner somewhat parallel to that of the type-specific capsular polysaccharide of Pneumococcus. It reacts well in the precipitin test with type-specific antisera, and by this means a serologically distinct substance can be demonstrated for each individual type. The M antibody produced by animals in response to immunization with intact living or dead streptococci or with solutions of the M substance extracted from them appears responsible for type-specific protection. In most types, the M antibody causes agglutination of M-containing organisms of the same type. In at least one type, however (Type 1), M antibody fails to bring about agglutination in spite of the fact that both M antigen and its antibody are present in the system and can be demonstrated by the precipitin reaction and by passive protection tests (81).

There is, at present, no explanation for this failure of the M antibody to cause agglutination of Type 1 organisms when all conditions seem favorable for the reaction. It is well-recognized that occasional strains belonging to various types may at times be refractory to agglutination but this inagglutinability is not comparable to the phenomenon described here, since Type 1 streptococci invariably fail to be agglutinated by M antibody. That the suspensions are agglutinable in the presence of an appropriate antibody is shown when they are brought into contact with serum containing a second type-specific antibody directed against the recently discovered, so-called, T substance. Prompt and striking type-specific agglutination then occurs.

Relation of the Type-specific Substance, T, to Agglutination. The most important *type-specific agglutinogen* in all types of Group A appears to be the T substance. Although antibody to this substance apparently has no protective action, it is probably the principal antibody responsible for type-classification based upon the agglutination reaction. This brings up the interesting question of the functioning of two type-specific substances in one microorganism. Unless M and T are definitely correlated, one could imagine many possible types due to many different antigenic combinations.

Correlation of M and T Antigens in Individual Types. Fortunately, while the number of types is indeed imposing, it is not often complicated by a lack of correspondence between the M and T antigens. Because the T antigen was unknown in 1934, when Griffith's report on twenty-three types of Group A hemolytic streptococci appeared, it was formerly thought that the same type-specific antibody was responsible for the M precipitin reaction and for the agglutination reactions, which Griffith was studying. Nevertheless, representative strains and sera[2] of Dr. Griffith's types were tested to make sure that this was true. For lack of suitable anti-M sera, not quite all types could be tested, but the few minor differences in results of type-determinations based on the M precipitin reaction or on the slide agglutination technique were easily reconciled with the single exception of Strain C203, a Group A streptococcus widely used for experimental work. For several years, it was a puzzle to understand why this strain appeared to be Type 1, as Dr. Griffith (43) classified it, by its agglutination reactions, and yet by the M precipitin reactions and mouse protection tests, studied in this laboratory, it reacted as Type 3. It finally became clear that this was due to an extra T substance not normally present in Type 3 streptococci. Thus, Strain C203 has both the M and T antigens of Type 3, and an additional T antigen of Type 1. The latter is the dominant antigen effective in the agglutination reactions of this strain (81).

From preliminary study, it seems probable that some such unusual association of antigens exists in certain other types which are hard to differentiate by their agglutination reactions. This seems true in the case of Types 17 and 23 (66, 69, 99), which some authors have thought identical. Their M substances are, however, as distinct as those of any other types, but some strains of these two types undoubtedly have a complex of T antigens not yet completely differentiated.

Another factor, complicating the analysis of immunological reactions of streptococci, is that the bacterial cells contain a

[2] These were generously supplied by Dr. Griffith.

mixture of nucleoproteins which are usually not type-specific, nor even species-specific in their serological reactions. Thus, in the precipitin reaction and also in active and passive anaphylaxis, these cellular components show marked cross relationships (72, 74–76). Reactions with them do not appear with all immune sera and all extracts. Rabbits vary in their response to immunization with whole streptococci, sometimes producing these so-called P-antibodies and sometimes failing to produce them. It has also been found that M extracts which usually give type-specific reactions may contain variable amounts of the nucleoprotein mixture, P. For these reasons, one must constantly be on the alert to avoid misinterpretation of results. Although it is not clear whether the P—anti-P systems are concerned in cross agglutinations, this possibility should always be borne in mind.

Characteristics of the Type-specific Protein, M. Before the discovery of the T substance, the protein, M, was subjected to careful study both of a serological and a chemical nature. This substance is obtained relatively free of other cellular proteins by extracting the streptococci with acid and heat, a procedure which renders most proteins insoluble; but the type-specific protein, M, is brought into solution by this treatment. The M substance flocculates near pH 4.2, and is precipitated from neutral solution by the addition of alcohol. It belongs, however, to the class of alcohol soluble proteins (38), exhibiting a number of chemical properties in common with these substances: for example, in the form of the hydrochloride, the M substance is soluble in 80 per cent alcohol. Another interesting peculiarity is the great rapidity with which the M substance is digested by trypsin and pepsin.

In spite of its protein nature, this substance first appeared to be a hapten, which was capable of giving *in vitro* reactions. It did, nevertheless, induce anaphylactic shock in guinea pigs passively sensitized with anti-M immune serum prepared against whole streptococci. At that time, however, it was not found antigenic when injected into rabbits and mice, nor did it actively sensitize guinea pigs.

Recently, the work of Stamp and Hendry (123), and that of Mudd and his associates (93, 140) has indicated that the M substance in more gently treated preparations might be a true antigen. Dr. Hirst and I (58) also found that under better conditions of extraction, the M substance is antigenic; and even in the form originally prepared and thought non-antigenic (73, 74, 76), it was subsequently found to exhibit a certain degree of antigenicity if larger doses were injected. When isolated from the cell, this substance is, however, a much poorer antigen than are the intact cells from which it was derived.

Proteolytic Digestion of the Type-specific M Substance from the Living Cell. Because proteolytic enzymes digest the type-specific protein, M, in solutions, the effect of enzymes on living hemolytic streptococci has been studied. Following Allison's suggestion, several investigators have treated streptococci with trypsin in order to transform granular, spontaneouly agglutinating cultures into diffuse, stable suspensions suitable for slide agglutination reactions (66). It was observed, however, both here and elsewhere (65), that these diffuse suspensions became at the same time very susceptible to agglutination with sera known to contain streptococcus agglutinins of a non-type-specific nature. This seemed analogous to the change occurring in a given strain of streptococcus when the matt form is degraded to the glossy by the loss of its type-specific protein, M (83, 134). This possibility was tested by trying to extract the M substance with hydrochloric acid from matt organisms which had been subjected to digestion with trypsin, or to obtain it from cultures grown in a medium containing active trypsin. It was found that the M substance could no longer be isolated from streptococci which had been treated in either of these ways with trypsin. The following facts were also observed: (1) The bacteria were not killed by digestion. Plate counts of suitable dilutions of the trypsin-treated and control cultures showed no significant difference in the number of organisms in each. (2) [3]Trypsin-treated streptococci were just as virulent for mice as the control cultures, appar-

[3] Some of these experiments were caried out by Dr. G. K. Hirst.

ently because the function of producing the M protein had not been affected. Consequently, when the first growth occurred in the peritoneal cavity, this protein was again produced. (3) [3]Bacteria, heat-killed immediately after treatment with trypsin, failed to induce the formation of M antibody when injected into rabbits, although the controls did so uniformly. (4) Other proteolytic enzymes were also able to digest M substance from the living bacteria in the same way. These experiments will be reported in full at another time. From this evidence, one must conclude that the M substance in the living bacteria is readily accessible to the action of proteolytic enzymes without injury to other vital functions of the living cells. This may be due to its possible location near the outer surface of the streptococci.

The Type-specific Substance, T, of Group A Streptococci. Investigation of the chemical nature and immunological activity of the second type-specific component of Group A hemolytic streptococci has been greatly hampered by the failure so far to recover it from the bacterial cell bodies in a form suitable for study.

Pathogenicity of Members of Different Types in Group A. Many investigators, studying the specific types of Group A hemolytic streptococci in different diseases, have found a lack of correlation between any particular types and the diseases in which they are found. Thus, scarlet fever, erysipelas, puerperal sepsis and other streptococcal infections may all be caused by the same types, or by different types at different times. Allison (1) stated in 1938, that the first four serological types included upwards of 50 per cent of all cases of scarlet fever occurring in London over a period of ten years. In Australia, on the other hand, a different series of types has predominated and these were not the same in Sydney, Melbourne or Adelaide (66). In 1935–6, in New York, Pauli and Coburn (99) found Types 4, 13 and 22 most common in throats of rheumatic fever patients; while Kuttner and Krumweide (70), in another group of rheumatic fever patients near New York in 1937–40, found still other types predominant in different years. Strains isolated from rheumatic fever and other

patients in the Rockefeller Hospital have likewise varied greatly from year to year. There is, apparently, no fixed relationship between the virulence of a type in one epidemic or locality and its effect at another time and place; and all gradations in severity of clinical manifestations may result from a single type in any one epidemic. Consequently, it does not seem possible that the type-specific substances, M and T, equally present on different occasions could be responsble for the divergent clinical effects, but rather that other unknown fluctuating factors affecting virulence must be operative in both host and parasite.

It has been demonstrated that the secondary complications in scarlet fever are often due to types different from those responsible for the primary attacks (2, 14, 136, 137). These secondary complications apparently arise from cross infection in wards containing patients carrying many different types. It is, therefore, indicated that, when individual isolation is impossible, patients placed in a single ward should be restricted to those carrying the same serological type of hemolytic streptococcus. When this has been done, the number of secondary streptococcal complications has been materially reduced. Hare's recent work on the carrier problem also lends weight to these observations (48–51).

Serological Types in Group B. The existence of specific types in Group B was first established by Stableforth (121) in England, who found three serological types among strains from bovine mastitis which were sharply defined by their precipitin and agglutinin reactions. Reasoning by analogy with the type-specific protein of Group A, he assumed that the type-specific substance in Group B was also a protein. It has developed from studies in our laboratory (78, 80), however, that the type-specific substances in this group are polysaccharides. We also differentiated four specific types on the basis of precipitin reactions with type-specific polysaccharides obtained respectively from each individual type, and different from the group-specific carbohydrate C. Sufficiently detailed chemical studies were made of the type-specific substances from two of these types to indicate

that they were polysaccharides. In the other less well-studied types, preliminary investigation indicated that polysaccharides were also responsible for type-specificity.

Correlation of antibodies against these polysaccharides with type-specific immunity was shown in the protection afforded mice infected with Types Ia or Ib, if the respective antisera were administered, and in the lack of such protection in the presence of antisera of heterologous Group B types. The importance of these polysaccharides in virulence was also shown by a million-fold decrease in mouse virulence when virulent microorganisms were so treated that they no longer elaborated type-specific polysaccharides. On immunization with these degraded cultures, type-specific precipitins and protective antibodies were not formed; but precipitin for the group-specific polysaccharide, C, was formed much more readily than usual and in greater amount.

These observations indicate that type-specific polysaccharides have a direct relationship to the antigenic properties of Group B streptococci, and in many instances appear to have an association with their pathogenicity.

English and Australian investigators (94, 117, 122, 124) have been particularly active in epidemiological studies involving the further differentiation of Group B types, but have not identified the antigens involved. Employing a slide agglutination technique, they have distinguished about fourteen main and subtypes among bovine strains, and five more types which included 86 out of 100 strains of human origin. Since only one of these 100 human strains fell into a bovine type, Simmons and Keogh (117) concluded that types of Group B streptococci indigenous to man and cattle are quite distinct and that human carriers are not, therefore, usually a source of infection in cattle. Brown (12, 13) and also Little (84) in this country, however, have indicated that the human carrier should be considered in attempts to eradicate bovine mastitis. My own study of a limited number of strains would indicate a somewhat greater frequency of the same specific types from human and bovine sources than that found by Simmons and Keogh (see Table VII). The difference in opinion

may possibly be due to the different methods used for identifying strains. Further study of this question is needed.

TABLE VII

Serological Types of Group B Streptococci Identified by Precipitin Reaction

Source	Number in Types			
	Ia	Ib	II	III
Human				
Non-pathogenic	6	14	8	20
Associated with disease	3	3	1	2
Etiological agent of disease*	1	1	3	6
Total human	10	18	12	28
Bovine				
Milk and mastitis	5	9	11	9
Reported by Plastridge and Hartsell (102)	28	1	9	4
Total bovine	33	10	20	13

* Includes strains from some of the fatal infections recorded in Table III.

Serological Types in Other Groups: Some progress has been made in several laboratories in distinguishing types within the other known groups.

Group C. The first serological types differentiated in Group C were the Types 7, 20, and 21 of Griffith's 1934 classification of *S. pyogenes* (43). These types are composed of large matt colony Group C strains that are probably similar culturally to the Types B1 and B2 of animal origin classified by Ogura by means of fermentation and other biochemical reactions. By using the fermentation of trehalose and sorbitol as differential tests, P. R. Edwards (28, 29), following the work of Ogura (97), has done much toward clarifying the differences between these so-called "human" Group C strains and the chief "animal" Group C strains (Type A Ogura). One would like to know how much connection there is between types determined by biochemical reactions and those based upon serological reactions. Evans has

pointed out relationships between certain series of serological types in Group A and susceptibility to the action of different races of bacteriophage which she has studied. Australian investigators also have concluded that definite correlation occurs between biochemical and serological reactions, at least in Groups A, C and G (65).

The latter investigators have attempted to classify into serological types the Group C strains encountered in their studies: on the one hand, strains chiefly from the human throat, and on the other, strains from equine infections. Using slide agglutinations, Simmons and Keogh found five main types among human throat strains; and Bazeley and Battle (5) found five main equine serological types which were in close agreement with the biochemical types, corresponding also to the biochemical types described previously by Ogura. Serological subtypes have been found, but not completely studied as yet within the main equine types. Most of these types have not been tested by means of the precipitin reaction.

Study of type-specific substances among Group C strains has not progressed very far. From at least one of the Group C types identified by Griffith (Type 21), I have, in preliminary experiments, obtained a type-specific substance which was digested by trypsin. No similar substance could be obtained from the mucoid animal strains (Type A, Ogura), although Stamp and Hendry (123) protected mice against infection with such a strain by immunizing them with a protein fraction obtained in extracts.

A type-specific substance was recently found in Group C strains of one specific type which caused nine separate out-breaks of suppurative arthritis in lambs in East Anglia, England (7). From extracts of these streptococci, a type-specific protein was separated from the group-specific polysaccharide, C. Lambs which recovered were immune and their blood contained a specific bactericidal substance. A further interesting feature was the demonstration that these Group C streptococci were the methemoglobin producing, or *alpha* type, similar to the *alpha* Group C strains from bovine mastitis. The authors draw atten-

tion to the similarity of the streptococci from joint-ill to these bovine strains and to the Group C strains of human origin. With none of the other human or animal Group C strains at their disposal were they able to induce joint-ill in lambs but none were of the same serological type as those from joint-ill.

Groups F and G. Both the precipitin and slide agglutination techniques have been applied by Bliss (8) with equal success to the differentiation of serological types among the "minute" hemolytic streptococci of Groups F and G. In Group F, two large and well-defined types have been found, and a third smaller and less distinct type seemed to be present. The "minute" strains belonging to Group G form one serological type, with a type-specific antigen which is so similar serologically to that of the Type 1, Group F, strains, as to suggest that these antigens may be identical chemically.

As already implied, these Type 1 strains of Groups F and G differ sharply in their group-specific antigens, the "C" polysaccharides, which are entirely characteristic of their respective groups (77). Serological relationships of this sort, based on chemical similarity of the antigens concerned, are not uncommon among bacteria, or even among apparently unrelated forms of life.

Simmons and Keogh (117), using slide agglutinations only, have investigated the serological types of 78 Group G strains of human origin isolated in Australia, and have found evidence of more than one type in the "minute" strains studied by them, and of three types among the large colony strains.

Miscellaneous Information Concerning Types in Various Groups. In our own laboratory, some evidence of the occurrence of specific types based on the precipitin reaction has been encountered among other groups as follows: Group D, 3 distinct types; Group E, 1; Group F, 2; Group G, 1; Group K, 1. Strains unclassified as to type remain in all groups. Houston and his collaborators have also indicated the finding of specific types among the enterococci (Group D) although the details have not, I think, been published (39, 61, 62).

In none of the types enumerated among groups other than A and B has the type-specific substance been isolated and studied except in crude extracts. Their behavior, however, suggests that the type-specific antigens in all except Groups A and C, are probably polysaccharides, but further study of these substances is needed to determine their chemical nature.

Overlapping Immunological Relationships. Overlapping immunological reactions have been observed with increasing frequency not only among bacteria but also in other natural sources widely separated generically. Although such cross reactions usually indicate genetic relationships, it also sometimes happens that no consistent relationship is apparent. However, where chemical data are available, it has been found that serological similarities are always based on chemical likenesses, whether the determinative substances are derived from related or unrelated sources.

Examples of this phenomenon are fairly common among hemolytic streptococci, and these cases suggest genetic relationships. These overlapping reactions are comprehensible because related chemical components have been isolated from the streptococci in several of these instances. Frequently, the overlapping specificity appears dependent on chemical relationships between type-specific polysaccharides, as between two types in Group B (78, 80). Another example occurs between two types, one of which is in Group F, the other in Group G (8). More obscure cross relationships, not always reciprocal, have been observed. These include agglutinations of certain Group B and C strains in sera of certain Group A types. Finally, some of the cross agglutinations in Group A which were formerly confusing are now understood because of knowledge concerning the T substance.

Summary of Group and Type Substances. In order to leave with you a clear conception of the Group and Type antigens so far known, I have prepared a table showing somewhat arbitrarily the simpler relationships of these substances. The first and second columns indicate that each group has an individual polysaccharide, the C substance, specific for that group. The last three columns show the substances concerned with type-speci-

TABLE VIII

Group and Type Specific Antigens of Hemolytic Streptococci

Group specificity		Type specificity			
Serological group	Group specific "C" substances	Specific types recognized	Type specific substances		
			Designated	Chemical nature	
A	Polysaccharides immunologically distinct for each group	At least 30	"M"	Proteins	Substances chemically and immunologically distinct for each type
			"T"	Undetermined	
B		Several	"S"	Polysaccharides	
C		Several	No symbol	Proteins	
Remaining groups		Several	"S"	Polysaccharides	

ficity within each group and what is known of their chemical nature. Thus, in each of the 30 odd types of Group A streptococci, an individual type-specific protein, M, is associated with virulence; and the antibodies to which this type-specific substance gives rise are responsible for the serological and protective action of immune sera. The second type-specific substance, T, in Group A, is known chiefly as an agglutinogen, usually one T substance for each type; its chemical nature is not yet determined. In Group B, the type-specific substance of each individual type is a polysaccharide, designated S, and appears to be solely responsible for type-specificity. In at least one type within Group C, a protein is the type-specific substance, and proteins also appear to be the type-specific substances in two other types within this group. In mucoid strains of this group, the nonantigenic mucoid polysaccharide plays an important part in virulence, but is not apparently associated with type-specificity.

In other groups, less is known about the type-specific substances, but the evidence suggests that polysaccharides are chiefly concerned.

SUMMARY

In summarizing the results of these immunochemical studies of hemolytic streptococci, one might question the wisdom of dissecting the microorganism so completely rather than studying its functions as a whole. In complicated systems where the interactions between bacteria and host are being investigated, much information can be obtained by studying the various components separately.

At present, the biological function of the group-specific polysaccharide C is not understood. Although we are unaware of how this substance contributes to the metabolism of the bacterial cell, it is useful as a means of identifying the origin of the particular streptococcus from which it was derived. In this way, strains pathogenic for man can be distinguished from those which are of animal origin and only rarely, if ever, induce disease in human beings. Other related characteristics can also be surmised by identifying this particular cell component.

The function of the type-specific substance is better understood than is that of the group-specific polysaccharides. It seems certain that the type-specific protein, M, is related to the virulence of Group A streptococci; and the host's reaction in producing antibodies against the M substance is one of the important means of protection against infection with these cocci. The type-specific activities of Group A strains are chiefly referable to the M protein, except that type-specific agglutination is now known to be related primarily to another cellular component of unknown chemical composition, the T substance. Antibodies engendered by this substance apparently do not exert any protective action in combatting infection. Whether the M and T substances exist in combined or separate form in the cell is still to be determined; but the presence of T substance, both in matt variants and their avirulent glossy derivatives, explains why Group A hemolytic streptococci may lose their type-specific M protein and still exhibit type-specific agglutination. Before the T component of the type-specific complex was known, it was difficult to explain the type-specific agglutination of strains producing little, if any, M protein. Even though not all of the immunological reactions of hemolytic streptococci are understood, the general pattern has become much clearer since this part of it has been elucidated.

The examples mentioned indicate the direction of these studies of hemolytic streptococci and emphasize the relationship of specific cellular components to the biological activities of the whole microorganism.

BIBLIOGRAPHY

1. Allison, V. D., *Lancet*, 1938, **1**, 1067.
2. Allison, V. D., and Brown, W. A., *J. Hyg.*, 1937, **37**, 11.
3. Barnard, W. G., and Todd, E. W., *J. Path. and Bact.*, 1940, **51**, 43.
4. Bazeley, P. L., *Austral. Vet. J.*, 1940, **16**, 187, 243.
5. Bazeley, P. L., and Battle, J., *Austral. Vet. J.*, 1940, **16**, 140.
6. Besredka, A., *Ann. Inst. Pasteur*, 1901, **15**, 880.
7. Blakemore, F., Elliott, S. D., and Hart-Mercer, J., *J. Path. and Bact.*, 1941, **52**, 57.
8. Bliss, E. A., *J. Bact.*, 1937, **33**, 625.
9. Bliss, E. A., and Long, P. H., *New Eng. J. Med.*, 1937, **217**, 18.
10. Boisvert, P. L., *J. Bact.*, 1940, **39**, 727.

11. Brown, J. H., Monographs of the Rock. Inst. for Med. Res., 1919, No. 9, New York.
12. Brown, J. H., *Cornell Vet.*, 1937, **27**, 110.
13. Brown, J. H., *J. Bact.*, 1939, **37**, 133.
14. Brown, W. A., and Allison, V. D., *J. Hyg.*, 1935, **35**, 283; 1937, **37**, 1.
15. Cannon, A. B., Sanders, M., and Rankin, J. L., *Arch. Dermatol. and Syphilol.*, 1940, **42**, 884.
16. Coburn, A. F., and Pauli, R. H., *J. Exp. Med.*, 1935, **62**, 129, 137, 159; 1941, **73**, 551.
17. Coffey, J. M., *J. Immunol.*, 1938, **35**, 121.
18. Colebrook, D. C., Med. Res. Coun. Spec. Rep., No. 205, London, H. M. Stationery Office, 1935.
19. Colebrook, L., and Purdie, A. W., *Lancet*, 1937, **2**, 1237, 1291.
20. Congdon, P. M., *Lancet*, 1935, **2**, 1287.
21. Cumming, W. M., *J. Path. and Bact.*, 1927, **30**, 279.
22. Davis, L. J., and Guzdar, J. S., *J. Hyg.*, 1935, **35**, 161.
23. Davis, L. J., and Guzdar, J. S., *J. Path. and Bact.*, 1936, **43**, 197.
24. Dawson, M. H., Hobby, G. L., and Olmstead, M., *J. Inf. Dis.*, 1938, **62**, 138.
25. Dochez, A. R., Avery, O. T., and Lancefield, R. C., *J. Exp. Med.*, 1919, **30**, 179.
26. Edwards, P. R., *J. Bact.*, 1932, **23**, 259.
27. Edwards, P. R., *J. Bact.*, 1933, **25**, 527.
28. Edwards, P. R., *J. Bact.*, 1934, **27**, 527.
29. Edwards, P. R., *Kentucky Agri. Exp. Sta. Bull.*, Mar., 1935, No. 356, p. 3.
30. Edwards, S. J., *J. Comp. Path. and Therap.*, 1932, **45**, 43.
31. Elser, W. J., and Thomas, R. A., *J. Bact.*, 1936, **31**, 79.
32. Evans, A. C., *Pub. Health Rep.*, U. S. P. H. S., 1934, **49**, 1386.
33. Evans, A. C., *J. Bact.*, 1940, **40**, 215.
34. Francis, A. E., *Brit. Med. J.*, 1941, **1**, 288.
35. Fry, R. M., *J. Path. and Bact.*, 1933, **37**, 337.
36. Fry, R. M., *Lancet*, 1938, **1**, 199.
37. Gay, F. P., *J. Lab. and Clin. Med.*, 1918, **3**, 721.
38. Goadby, K., *Proc. Roy. Soc., London*, 1927–8, **102**, 137.
39. Graham, N. C., and Bartley, E. O., *J. Hyg.*, 1939, **39**, 538.
40. Griffith, F., *J. Hyg.*, 1926, **25**, 385.
41. Griffith, F., *J. Hyg.*, 1927, **26**, 363.
42. Griffith, F., *J. Hyg.*, 1928, **27**, 113.
43. Griffith, F., *J. Hyg.*, 1934, **34**, 542.
44. Gunnison, J. B., Luxen, M. P., Cummings, J. R., and Marshall, M. S., *J. Bact.*, 1940, **39**, 689.
45. Gunnison, J. B., Luxen, M. P., Marshall, M. S., and Engle, B. Q., *J. Dairy Sci.*, 1940, **23**, 447.

46. Hare, R., *J. Path. and Bact.*, 1935, **41**, 499.
47. Hare, R., *J. Path. and Bact.*, 1937, **44**, 71.
48. Hare, R., *Canad. Pub. Health J.*, 1937, **28**, 1.
49. Hare, R., *Canad. Pub. Health J.*, 1940, **31**, 407.
50. Hare, R., *Canad. Pub. Health J.*, 1940, **31**, 539.
51. Hare, R., *Lancet*, 1940, **1**, 109.
52. Hare, R., and Maxted, W. R., *J. Path. and Bact.*, 1935, **41**, 513.
53. Hare, T., and Fry, R. M., *Vet. Rec.*, 1938, **50**, 213.
54. Heidelberger, M., and Avery, O. T., *J. Exp. Med.*, 1923, **38**, 73; 1924, **40**, 301.
55. Heidelberger, M., and Kendall, F. E., *J. Immunol.*, 1936, **30**, 267.
56. Hill, A. M., and Butler, H. M., *Med. J. Austral.*, 1940, **1**, 222, 293.
57. Hirst, G. K., *J. Exp. Med.*, 1941, **73**, 493.
58. Hirst, G. K., and Lancefield, R. C., *J. Exp. Med.*, 1939, **69**, 425.
59. Hitchcock, C. H., *J. Exp. Med.*, 1924, 40, 445.
60. Hitchcock, C. H., *J. Exp. Med.*, 1924, 40, 575.
61. Houston, T., *Ulster Med. J.*, 1934, **3**, 224.
62. Houston, T., Second Internat. Cong. for Microbiology, London, 1936, Rep. of Proc., 141.
63. Keefer, C. S., and Spink, W. W., *J. Clin. Invest.*, 1937, **16**, 155.
64. Kendall, F. E., Heidelberger, M., and Dawson, M. H., *J. Biol. Chem.*, 1937, **118**, 61.
65. Keogh, E. V., and Simmons, R. T., *J. Path. and Bact.*, 1940, **50**, 137.
66. Keogh, E. V., Simmons, R. T., and Wilson, H., *Aust. J. Exp. Biol. and Med. Sci.*, 1941, **19**, 51.
67. Kobayashi, R., Dept. of Bacteriology, Medical College, Keio-Gijuku University, Tokyo, 1939.
68. Kodama, T., *Kitasato Arch. Exp. Med.*, 1936, **13**, 101, 217.
69. Kodama, T., Ozaki, M., Nishiyama, S., and Chiku, Y., *Kitasato Arch. Exp. Med.*, 1938, **15**, 162.
70. Kuttner, A. G., and Krumweide, E., *J. Clin. Invest.*, 1941, **20**, 273.
71. Lancefield, R. C., *J. Exp. Med.*, 1925, **42**, 377.
72. Lancefield, R. C., *J. Exp. Med.*, 1925, **42**, 397.
73. Lancefield, R. C., *J. Exp. Med.*, 1928, **47**, 91.
74. Lancefield, R. C., *J. Exp. Med.*, 1928, **47**, 469.
75. Lancefield, R. C., *J. Exp. Med.*, 1928, **47**, 843.
76. Lancefield, R. C., *J. Exp. Med.*, 1928, **47**, 857.
77. Lancefield, R. C., *J. Exp. Med.*, 1933, **57**, 571.
78. Lancefield, R. C., *J. Exp. Med.*, 1934, **59**, 441.
79. Lancefield, R. C., *J. Exp. Med.*, 1934, **59**, 459.
80. Lancefield, R. C., *J. Exp. Med.*, 1938, **67**, 25.
81. Lancefield, R. C., *J. Exp. Med.*, 1940, **71**, 521, 539.
82. Lancefield, R. C., and Hare, R., *J. Exp. Med.*, 1935, **61**, 335.
83. Lancefield, R. C., and Todd, E. W., *J. Exp. Med.*, 1928, **48**, 769.

84. Little, R. B., *J. Exp. Med.*, 1938, **68**, 905.
85. Little, R. B., *Proc. Soc. Exp. Biol. and Med.*, 1939, **41**, 254.
86. Loewenthal, H., *Brit. J. Exp. Path.*, 1938, **19**, 143.
87. Long, P. H., and Bliss, E. A., *J. Exp. Med.*, 1934, **60**, 619.
88. Mandelbaum, M., *Z. Hyg. u. Infectionskrankh.*, 1907–8, **58**, 26.
89. MacCallum, W. G., and Hastings, T. W., *J. Exp. Med.*, 1899, **4**, 521.
90. Macdonald, I., *Med. J. Aust.*, 1939, **2**, 471.
91. McLeod, J. W., *J. Path. and Bact.*, 1911–12, **16**, 321; 1914–15, **19**, 392.
92. Miller, W. T., and Heishman, J. O., *Cornell Vet.*, 1940, **30**, 310.
93. Mudd, S., and Lackman, D. B., *J. Immunol.*, 1940, **39**, 495.
94. Murnane, D., Commonwealth of Australia, Bull. No. 134, Melbourne, 1940.
95. Neill, J. M., and Mallory, T. B., *J. Exp. Med.*, 1926, **44**, 241.
96. Neter, E., and Young, G. S., *Am. J. Dis. Children*, 1937, **53**, 1531.
97. Ogura, K., *J. Jap. Soc. Vet. Sci.*, 1929, **8**, 174.
98. Pasteur, L., *Bull. de l'Acad. de Med.*, 1879, **8**, 256, in Oeuvres de Pasteur, P. Vallery Radot, 1933, **6**, 131.
99. Pauli, R. H., and Coburn, A. F., *J. Exp. Med.*, 1937, **65**, 595.
100. Pilot, I., Buck, C., and Davis, D. J., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 233.
101. Pilot, I., Buck, C., Davis, D. J., and Eastman, D. A., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 499.
102. Plastridge, W. N., and Hartsell, S. E., *J. Inf. Dis.*, 1937, **61**, 110.
103. Plummer, H., *J. Bact.*, 1934, **27**, 465.
104. Plummer, H., *J. Bact.*, 1935, **30**, 5.
105. Pomales-Lebrón, A., *Puerto Rico J. Pub. Health & Trop. Med.*, 1940, **16**, 66.
106. Rantz, L. A., and Keefer, C. S., *J. Inf. Dis.*, 1941, **68**, 128.
107. Schottmüller, H., *Munch. Med. Woch.*, 1903, **1**, 849.
108. Seastone, C. V., *J. Bact.*, 1934, **28**, 481.
109. Seastone, C. V., *J. Exp. Med.*, 1939, **70**, 347, 361.
110. Seegal, B. C., Heller, G., and Jablonowitz, J., *Proc. Soc. Exp. Biol. and Med.*, 1936, **34**, 812.
111. Sherman, J. M., *Bact. Rev.*, 1937, **1**, 1.
112. Sherman, J. M., *J. Bact.*, 1938, **35**, 81.
113. Sherman, J. M., and Niven, C. F., *J. Inf. Dis.*, 1938, **62**, 190.
114. Sherman, J. M., Smiley, K. L., and Niven, C. F., *J. Dairy Sci.*, 1940, **23**, 529.
115. Sherman, J. M., and Stark, P., *J. Dairy Sci.*, 1934, **17**, 525.
116. Sherman, J. M., Stark, P., and Mauer, J. C., *J. Bact.*, 1937, **33**, 483.
117. Simmons, R. T., and Keogh, E. V., *Austral. J. Exp. Biol. and Med. Sci.*, 1940, **18**, 151.
118. Smith, F. R., Niven, C. F., and Sherman, J. M., *J. Bact.*, 1938, **35**, 425.

119. Smith, T., and Brown, J. H., *J. Med. Research,* 1914–15, **31**, N.S. **26**, 455.
120. Smythe, C. V., and Harris, T. N., *J. Immunol.,* 1940, **38**, 283.
121. Stableforth, A. W., *J. Comp. Path. and Therap.,* 1932, **45**, 185.
122. Stableforth, A. W., *J. Path. and Bact.,* 1937, **45**, 263.
123. Stamp, T. C., and Hendry, E. B., *Lancet,* 1937, **1**, 257.
124. Stewart, D. F., *J. Path. and Bact.,* 1937, **45**, 279.
125. Tillett, W. S., *Bact. Rev.,* 1938, **2**, 161.
126. Todd, E. W., *Brit. J. Exp. Path.,* 1928, **9**, 1.
127. Todd, E. W., *J. Exp. Med.,* 1928, **48**, 493.
128. Todd, E. W., *J. Exp. Med.,* 1932, **55**, 267.
129. Todd, E. W., *J. Path. & Bact.,* 1934, **39**, 299.
130. Todd, E. W., *Brit. J. Exp. Path.,* 1938, **19**, 367.
131. Todd, E. W., *J. Path. & Bact.,* 1938, **47**, 423.
132. Todd, E. W., *J. Hyg.,* 1939, **39**, 1.
133. Todd, E. W., Coburn, A. F., and Hill, A. B., *Lancet,* 1939, **2**, 1213.
134. Todd, E. W., and Lancefield, R. C., *J. Exp. Med.,* 1928, **48**, 751.
135. Torrey, J. C., and Montu, E., *J. Inf. Diseases,* 1934, **55**, 340.
136. de Waal, H. L., *J. Hyg.,* 1940, **40**, 172.
137. de Waal, H. L., *J. Hyg.,* 1941, **41**, 65.
138. Weld, J. T., *J. Exp. Med.,* 1934, **59**, 83.
139. Weld, J. T., *J. Exp. Med.,* 1935, **61**, 473.
140. Zittle, C. A., *J. Immunol.,* 1939, **37**, 1.