STUDIES ON THE BIOLOGY OF STREPTOCOCCUS.

I. Antigenic Relationships between Strains of Streptococcus hæmolyticus.

By A. R. DOCHEZ, M.D., O. T. AVERY, M.D., and R. C. LANCEFIELD.

(From the Hospital of The Rockefeller Institute for Medical Research.)

(Received for publication, June 1, 1919.)

The importance of the problem of the systematic classification of bacteria for the proper understanding and control of infectious diseases is becoming increasingly evident. Such study is necessary not only in the elucidation of the biological relationship existing between varieties of the same species of bacterium, but is also essential to the working out of epidemiological problems and to the development of knowledge useful in the effort to control infectious diseases by means of specific therapeutic and prophylactic measures. Bacteria closely resembling those responsible for the pathological process in many acute infections have been found to be present and to live, apparently without harm to the host, on the mucous membranes of a large proportion of normal individuals. The resemblance of the pathogenic to the harmless variety of microorganism is frequently so close that in many instances tests of particular specificity are required to show the existing biological differences. In fact, the problem in etiology today is to determine not only the bacterial species causing a given disease, but in addition the number of varieties of the same species that are pathogenic, and whether common and important non-pathogenic varieties exist. The study is one of varying complexity, and methods suitable to one species do not give the desired information when applied to another.

The purpose of such studies may be broadly defined as an effort to relate fixed and determinable characteristics of bacteria to pathogenicity. Though fluctuating variations of bacteria probably occur, it seems not unlikely that in most diseases a sufficiently constant equilibrium has been attained to justify the usefulness of the effort. The

methods employed in these studies are numerous. In some species morphological and cultural characters give important though somewhat limited information, and in many the biochemical reactions are of great significance. The most serviceable method, however, for obtaining the particular kind of knowledge desired is the study of immunological relationships. For some as yet unexplained reason the latter specific reactions are very constant among the pathogenic varieties. In the following study of *Streptococcus hæmolyticus* chief dependence has been placed on the knowledge obtained from the study of these immunity reactions.

In the various classifications of the streptococcus group as a whole that have been proposed, the custom has been to consider the strains that effect hemolysis of red blood cells as constituting a single or unit type (1). The validity of this assumption has been questioned and there has been much study and discussion of the probability of the existence of variations within this group, some evidence of which has been obtained from the study of sugar fermentations (2). Recent studies (3) indicate that certain broad lines of differentiation may be shown between hemolytic streptococci of human origin, and those of bovine origin whether found in milk or cheese. Hemolytic streptococci of the human type are usually found in association with some pathological process such as puerperal sepsis, septicemia, erysipelas, bronchopneumonia, or other conditions, and hemolytic activity is generally considered as an indication of pathogenicity. With the development of our knowledge (4), however, these organisms have been found with increasing frequency when no pathological lesion has been apparent. Investigators for many years have been interested in these strains, and discussion has centered about the unity or multiplicity of the group (5). The evidence in general favors the belief that hemolytic streptococci pathogenic for human beings comprise a single type. In the present paper it will be shown that by the use of properly controlled immune reactions differential characters between individual strains can be shown to exist.

During the winter of 1917–18 in the United States, there occurred in numerous localities a great increase in the incidence of a previously rather infrequent type of bronchopneumonia. The highest morbidity rate and earliest appearance of this disease were in military cantonments. In the spring of 1918, however, the type of infection under consideration was commonly observed in the civilian population. The disease first appeared as a secondary pneumonia following measles, but soon instances of apparently primary infection of the lungs were observed. Numerous studies of the bacteriology

of this condition have demonstrated that the infectious agent responsible for the lung lesion was in almost every instance *Streptococcus hæmolyticus*. As a result of the widespread incidence of the disease the latter organism became extensively distributed and was frequently found as a secondary invader in acute lobar pneumonia, and as a common inhabitant of the normal throat.

The material used in the present study was obtained from the military establishments in the neighborhood of Fort Sam Houston, Texas. The sources of the individual strains were the throats of patients suffering from acute measles, the sputum of patients with bronchopneumonia both primary and secondary to measles, pathological material obtained from cases of bronchopneumonia and acute lobar pneumonia, and the throats of healthy individuals who had been directly or indirectly exposed to infection in a variety of ways.

All the strains of hemolytic streptococcus employed in this study possess the typical characteristics of the group. They are hardy organisms and grow readily in meat infusion broth and on blood agar slants. They survive for many months when grown for 18 hours in rabbit blood broth and subsequently placed at refrigerator temperature. In meat infusion broth the growth has been of two types —a granular sediment with clear supernatant fluid and a flocculent sediment with turbidity throughout the remainder of the tube. All the organisms are strongly Gram-positive, grow in chains of varying length, and are bile-insoluble. Capsule formation has not been observed. On plates two types of colonies are seen—a small, round, smooth colony and a moist ameboid colony with a slightly roughened surface. All the strains are actively hemolytic, showing a wide zone of hemolysis on the surface of rabbit blood agar plates; hemolysis is complete in 2 hours, when a 5 per cent suspension of washed rabbit blood cells suspended in salt solution is mixed with an equal quantity of a 24 hour broth culture. The power of the different strains to ferment the usual test substances for streptococcus has been studied. The majority fall in the group of *Streptococcus pyogenes* according to Holman's classification of streptococcus on a basis of sugar fermentations. None of the strains ferments inulin and about 20 per cent of these actively hemolytic strains possess the power of fermenting mannite. The latter characteristic will be shown to have an inter-

esting relationship to the immunological classification of these organisms.

The virulence of this group of hemolytic streptococci is low for the ordinary laboratory animals in comparison with such an organism, for instance, as pneumococcus. Doses of 1 cc. or more of a 24 hour broth culture administered intraperitoneally are required to kill guinea pigs and rabbits. Furthermore, repeated passages through these animals fail to bring about a considerable accession of virulence. The fatal dose for white rats and mice is smaller, usually in the neighborhood of 0.1 cc. of a broth culture. By continuous passage through rats and mice it has been possible to raise the virulence of a certain number of strains to a point where 0.001 cc. of broth culture is lethal for a rat in 24 hours and 0.00000001 cc. for a white mouse. On the other hand, many strains cannot be raised to this high degree of virulence even after the most persevering effort. Once the maximum of virulence is attained, this quality persists without renewed animal passages for an indefinite period of time.

The sources of the strains of *Streptococcus hæmolyticus* studied and some of their common characteristics are shown in Table I.

Although the finer differential classification of single species of bacteria by means of immune reactions is still in the earlier stages of development, enough evidence has been gathered to indicate that the more highly parasitic varieties of the species are more likely to consist of a limited number of unit types than are the less parasitic or the saprophytic members. In other words, unity of type seems to characterize the disease-producing microorganisms, whereas heterogeneity is more common among the non-pathogens. If this assumption is true it then becomes important to choose for purposes of classification the immune reactions which bring out most sharply the kind of differences sought, rather than a reaction which develops the basic relationship existing between all strains of the same species. For this purpose we regard the reactions of agglutination and protection as of superior usefulness to those of precipitation and complement fixation. The validity of any final classification arrived at depends, of course, upon the possibility of fitting accurately into such a classification a large number of strains freshly obtained from their natural environment.

TABLE I.

Source and Common Characters of Strains of Streptococcus hæmolyticus Studied.

Strain No.	Source.	Clinical diagnosis.	Hemolysis.	Sugar fermentations.				
				Lactose.	Salicin.	Mannite.	Inulin.	Raffinose.
		Type S 3.						
S 5	Autopsy (lung).	Bronchopneumonia following measles.	++++	+	+	−	−	−
S 29	Sputum.	" "	++++	+	+	−	−	−
S 114	"	" "	++++	+	+	−	−	−
S 118	Pleural fluid.	" "	++++	+	+	−	−	−
S 124	Sputum.	" "	++++	+	+	−	−	−
S 145	Throat.	" "	++++	+	+	−	−	−
S 146	Chest fluid.	" "	++++	+	+	−	−	−
S 149	Blood.	" "	++++	+	+	−	−	−
S 151	Pleural fluid.	" "	++++	+	+	−	−	−
S 2	Autopsy (blood).	Bronchopneumonia.	++++	+	+	−	−	−
S 3	" (lung).	"	++++	+	+	−	−	−
S 14	" "	"	++++	+	+	−	−	−
S 111	Chest fluid.	"	++++	+	+	−	−	−
S 53	Throat.	Measles.	++++	+	+	−	−	−
S 64	"	"	++++	+	+	−	−	−
S 154	"	"	++++	+	+	−	−	−
S 80	"	German measles.	++++	+	+	−	−	−
S 140	"	" "	++++	+	+	−	−	−
S 11	Sputum.	Lobar pneumonia (Type I).	++++	+	+	−	−	−
S 16	"	" "	++++	+	+	−	−	−
S 31	Throat.	" "	++++	+	+	−	−	−
S 44	"	" "	++++	+	+	−	−	−
S 83	"	" "	++++	+	+	−	−	−
S 95	"	" "	++++	+	+	−	−	−
S 125	"	" "	++++	+	+	−	−	−
S 144	"	" "	++++	+	+	−	−	−
S 8	"	Pneumonia.	++++	+	+	−	−	−
S 131	"	"	++++	+	+	−	−	−
S 41	"	Incipient tuberculosis.	++++	+	+	−	−	−

TABLE I—*Continued.*

Strain No.	Source.	Clinical diagnosis.	Hemolysis.	Sugar fermentations.				
				Lactose.	Salicin.	Mannite.	Inulin.	Raffinose.
		Type S 23.						
S 39	Autopsy (lung).	Bronchopneumonia following measles.	++++	+	+	−	−	−
S 78	Pleural fluid.	" "	++++	+	+	−	−	−
S 101	" "	" "	++++	+	+	−	−	−
S 107	Sputum.	" "	++++	+	+	−	−	−
S 27	Autopsy (blood).	Bronchopneumonia.	++++	+	+	−	−	−
S 67	Blood.	"	++++	+	+	−	−	−
S 120	Autopsy (blood).	"	++++	+	+	−	−	−
S 98	Throat.	Measles.	++++	+	+	−	−	−
S 116	"	"	++++	+	+	−	−	−
S 117	"	"	++++	+	+	−	−	−
S 9	"	Lobar pneumonia.	++++	+	+	−	−	−
S 23	"	" "	++++	+	+	−	−	−
S 56	Autopsy (lung).	" "	++++	+	+	−	−	−
S 65	Sputum.	" "	++++	+	+	−	−	−
S 75	Throat.	" "	++++	+	+	−	−	−
S 133	"	" "	++++	+	+	−	−	−
S 104	"	Pneumonia.	++++	+	+	−	−	−
S 130	"	"	++++	+	+	−	−	−
S 46	"	Incipient tuberculosis.	++++	+	+	−	−	−
S 122	"	" "	++++	+	+	−	−	−

TABLE I—*Continued.*

Strain No.	Source.	Clinical diagnosis.	Hemolysis.	Sugar fermentations.				
				Lactose.	Salicin.	Mannite.	Inulin.	Raffinose.
		Type S 60.						
S 137	Pleural fluid.	Bronchopneumonia following measles.	++++	+	+	+	−	−
S 6	Autopsy (lung).	Bronchopneumonia.	++++	+	+	+	−	−
S 35	Pleural fluid.	"	++++	+	+	+	−	−
S 55	Sputum.	"	++++	+	+	+	−	−
S 10	Throat.	Measles.	++++	+	+	+	−	−
S 37	"	"	++++	+	+	+	−	−
S 43	"	"	++++	+	+	+	−	−
S 60	"	"	++++	+	+	+	−	−
S 66	"	"	++++	+	+	+	−	−
S 71	"	"	++++	+	+	+	−	−
S 86	"	"	++++	+	+	+	−	−
S 88	"	"	++++	+	+	+	−	−
S 89	"	"	++++	+	+	+	−	−
S 100	"	"	++++	+	+	+	−	−
S 109	"	"	++++	+	+	+	−	−
S 123	"	"	++++	+	+	+	−	−
S 127	"	"	++++	+	+	+	−	−
S 128	"	"	++++	+	+	+	−	−
S 4	"	German measles.	++++	+	+	+	−	−
S 19	"	Lobar pneumonia.	++++	+	+	+	−	−
S 62	"	" "	++++	+	+	+	−	−
S 72	"	" "	++++	+	+	+	−	−
S 21	"	Incipient tuberculosis.	++++	+	+	+	−	−
S 141	"	" "	++++	+	+	+	−	−
S 150	"	" "	++++	+	+	+	−	−
S 267	Foot.	Cellulitis.	++++	+	+	+	−	−
S 269	Blood.	Erysipelas.	++++	+	+	+	−	−

TABLE I—*Continued.*

Strain No.	Source.	Clinical diagnosis.	Hemolysis.	Sugar fermentations.				
				Lactose.	Salicin.	Mannite.	Inulin.	Raffinose.
		Type S 84.						
S 1	Autopsy (lung).	Bronchopneumonia following measles.	++++	+	+	−	−	−
S 20	" (blood).	" "	++++	+	+	−	−	−
S 110	Sputum.	" "	++++	+	+	−	−	−
S 138	"	" "	++++	+	+	−	−	−
S 50	Pleural fluid.	Bronchopneumonia.	++++	+	+	−	−	−
S 84	" "	"	++++	+	+	−	−	−
S 139	Throat.	"	++++	+	+	−	−	−
S 115	"	German measles.	++++	+	+	−	−	−
S 15	"	Lobar pneumonia.	++++	+	+	−	−	−
		Unclassified.						
S 32	Autopsy (lung).	Bronchopneumonia following measles.	++++	+	+	−	−	−
S 59	" (blood).	" "	++++	+	+	−	−	−
S 93	Sputum.	" "	++++	+	+	−	−	−
S 97	Pleural fluid.	" "	++++	+	+	−	−	−
S 136	Autopsy (lung).	" "	++++	+	+	−	−	−
S 142	Sputum.	" "	++++	+	+	−	−	−
S 49	"	Bronchopneumonia following German measles.	++++	+	+	−	−	−
S 24	Autopsy (lung).	Bronchopneumonia.	++++	+	+	−	−	−
S 18	Throat.	Measles.	++++	+	+	−	−	−
S 26	"	"	++++	+	+	−	−	−
S 42	"	"	++++	+	+	−	−	−
S 51	"	"	++	+	+	−	−	+
S 63	"	"	++++	±	+	−	−	−
S 96	"	"	++++	+	+	−	−	−
S 102	"	"	++++	+	+	−	−	−
S 106	"	"	++++	+	+	−	−	−
S 108	"	"	++++	±	+	−	−	−
S 148	"	"	++++	+	+	−	−	−

TABLE I—*Concluded.*

Strain No.	Source.	Clinical diagnosis.	Hemolysis.	Sugar fermentations.				
				Lactose.	Salicin.	Mannite.	Inulin.	Raffinose.
		Unclassified—*Concluded.*						
S 17	Throat.	German measles.	+	−	−	+	−	−
S 47	"	" "	+++	+	−	−	−	−
S 48	"	" "	++++	+	+	−	−	−
S 54	Sputum.	" "	++++	+	+	−	−	−
S 68	Throat.	" "	++++	+	+	−	−	−
S 69	Autopsy (lung).	Lobar pneumonia.	++++	+	+	−	−	−
S 87	Sputum.	" "	++++	+	+	−	−	−
S 90	Throat.	" "	++++	+	+	−	−	−
S 99	Sputum.	" "	++++	+	+	−	−	−
S 34	Throat.	Incipient tuberculosis.	++++	+	+	+	−	+
S 36	"	" "	++++	+	+	−	−	−
S 121	"	" "	++++	+	−	−	−	−
S 129	"	" "	++++	±	+	+	−	−
S 155	"	" "	++++	+	+	−	−	−
S 264	Blood.	Osteomyelitis.	++++	+	+	−	−	−
S 271	"	Septicemia.	++++	+	+	−	−	−
S 272	Pus.	Abscess (measles).	++++	+	+	−	−	−
S 273	"	Scarlet fever.	++++	+	+	−	−	−
S 276	"	Pelvic abscess.	++++	+	+	−	−	−
S 277	" (abdomen).	Peritonitis.	++++	+	+	−	−	−
S 286	Pleural fluid.	Pneumonia.	++++	+	+	−	−	−
S 288	Sputum.	"	++++	+	+	−	−	−

The Reaction of Agglutination.

Specific agglutination has been found to be one of the most serviceable immune reactions for purposes of the biological classification of bacteria. In the typhoid and pneumococcus groups, for instance, it serves to distinguish clearly the different varieties from one another. It is likewise applicable to the classification of many other microorganisms. Efforts to classify the streptococci by means of this reaction apparently have not illuminated materially the relationship of one strain to another, nor have they shown a definite relationship between certain strains and a particular pathological process. A number of explanations of this fact may be proposed. In many instances no attention has been paid to the broader groupings of streptococci as determined by hemotoxin production and the fermentation of test sugars. Also streptococcus most frequently acts as a secondary invader in the production of disease, and it is probably an unwarranted assumption to suppose that type specificity is closely related to the character of the pathological process. One of the chief obstacles to the successful carrying out of the agglutination reaction has been the tendency of all types of streptococcus to undergo spontaneous granulation, and when used for tests to exhibit the phenomenon of non-specific agglutination. As a result of this, the reactions must usually be read against a more or less granular background, making it difficult, if not impossible, to distinguish between the non-specific and the specific influences. The tendency to spontaneous clumping is occasioned by several factors, only a few of which are understood. For instance, a homogeneous suspension of a granular streptococcus can easily be prepared by washing the organism several times with distilled water, and then resuspending in the same medium. The suspension will remain homogeneous for an indefinite period of time. If sodium chloride in concentrations above 0.06 per cent is added to the suspension, granulation immediately ensues. Many other salts act in the same manner. Substances antagonistic to this salt action may be added to the medium and function even to the extent of suspending the participation of the salt in the immune reaction, so that specific agglutination may be completely inhibited. Fortunately intermediate combinations can be found in which most

strains remain diffuse and in which the salt is still able to fulfill its part in the immune reaction. The most useful substance of this kind is ordinary meat infusion broth to which 1 per cent peptone has been added.[1] In addition, if streptococci are exposed to too great acidity the tendency to granulation is increased. In order to avoid this, the reaction of the medium may be so adjusted and such quantities of balanced phosphate solutions added that during growth an acidity greater than pH 7.1 is not attained. Certain other undetermined factors cause granulation, which may be defined as a general unsuitability of the medium for growth, and these can be eliminated only by experimenting with different preparations.

Technique.

The immune sera used in the agglutination and protection tests were obtained by the immunization of rabbits, sheep, and dogs. The animals were inoculated intravenously with repeated doses of heat-killed organisms, and in most instances a certain number of doses of living organisms was given. The employment for immunization of freshly isolated unpassed human strains, or the use of the same strains after a series of animal passages, does not alter in any recognizable way the specific qualities of the serum. The agglutinin and protective titer of the sera has remained undiminished for many months after the time of bleeding.

Great care is taken in the preparation of the organisms to be used in the agglutination reaction. The broth is made from carefully selected meat, and instead of the usual sodium chloride a sufficient quantity of a balanced phosphate mixture is added to give the required salt concentration and to adjust the hydrogen ion concentration at a pH of 7.4. When *Streptococcus hæmolyticus* is grown for 24 hours in a medium to which no sugar has been added, it does not develop an acidity greater than pH 7.2, which is just above the point at which the tendency to granulation appears. The organisms are removed from the culture medium by centrifugalization and are

[1] The authors are greatly indebted to Dr. Charles Krumwiede, Jr., of the Research Laboratories of the Department of Health of the City of New York, for many helpful suggestions in this technique.

washed once or twice in broth with a pH of 7.4 prepared in the manner described above. They are then resuspended in the same medium in approximately the concentration that is attained after 24 hours growth and are ready for use in the reaction. The sera to be employed in the tests are made up to the various concentrations by dilution with the same broth used for suspending the bacteria. The reaction of each specific serum is controlled by a complete series of like dilutions of normal serum of the same animal. Equal quantities of serum dilution and bacterial suspension are added to each tube and the tubes are then placed at a temperature of 55°C. for 1 hour. A temperature of 55°C. must be accurately maintained during the entire period and the tests must be read after the lapse of 1 hour, since if they are

TABLE II.

Power of Antistreptococcus Serum, Type S 3, to Agglutinate Ten Representative Strains of the Same Type.

Strain No.	Serum.	Dilution.									
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	Broth.
S 3	Type S 3	+±	+±	+±	+±	+±	+±	+±	+	±	−
	Normal.	−	−	−	−	−	−	−	−	−	
S 14	Type S 3	+±	+±	+±	+±	+±	+±	+±	+	±	−
	Normal.	−	−	−	−	−	−	−	−	−	
S 149	Type S 3	+±	+±	+±	+±	+±	+±	+±	+±	+±	−
	Normal.	±	±(?)	−	−	−	−	−	−	−	
S 95	Type S 3	++	++	++	++	++	++	++	++	+±	−
	Normal.	−	−	−	−	−	−	−	−	−	
S 80	Type S 3	+±	+±	+±	+±	+±	+±	+±	+±	+	−
	Normal.	−	−	−	−	−	−	−	−	−	
S 118	Type S 3	+±	+±	+±	+±	+±	+±	+±	+	±	−
	Normal.	−	−	−	−	−	−	−	−	−	
S 111	Type S 3	+±	+±	+±	+±	+±	+±	+±	+±	+	−
	Normal.	−	−	−	−	−	−	−	−	−	
S 140	Type S 3	+±	+±	+±	+±	+±	+±	+±	+±	+±	−
	Normal.	−	−	−	−	−	−	−	−	−	
S 16	Type S 3	+±	+±	+±	+±	+±	+±	+±	+±	+	−
	Normal.	−	−	−	−	−	−	−	−	−	
S 83	Type S 3	++	++	++	++	++	++	+±	+	±	−
	Normal.	−	−	−	−	−	−	−	−	−	

TABLE III.

Test of Cross-Agglutination Reactions of Antistreptococcus Serum, Type S 3, with Strains of Streptococcus hæmolyticus of Other Types and with Unclassified Strains.

Strain No.	Type of *S. hæmolyticus*.	Serum.	Dilution.									
			1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	Broth.
S 3	S 3	Type S 3	+±	+±	+±	+±	+±	+±	+±	+	±	−
		Normal.	−	−	−	−	−	−	−	−	−	
S 23	S 23	Type S 3	−	−	−	−	−	−	−	−	−	−
		Normal.	−	−	−	−	−	−	−	−	−	
S 107	S 23	Type S 3	−	−	−	−	−	−	−	−	−	−
		Normal.	−	−	−	−	−	−	−	−	−	
S 67	S 23	Type S 3	−	−	−	−	−	−	−	−	−	−
		Normal.	−	−	−	−	−	−	−	−	−	
S 65	S 23	Type S 3	−	−	−	−	−	−	−	−	−	−
		Normal.	−	−	−	−	−	−	−	−	−	
S 75	S 23	Type S 3	−	−	−	−	−	−	−	−	−	−
		Normal.	−	−	−	−	−	−	−	−	−	
S 128	S 60	Type S 3	−	−	−	−	−	−	−	−	−	−
		Normal.	−	−	−	−	−	−	−	−	−	
S 55	S 60	Type S 3	−	−	−	−	−	−	−	−	−	−
		Normal.	−	−	−	−	−	−	−	−	−	
S 60	S 60	Type S 3	−	−	−	−	−	−	−	−	−	−
		Normal.	−	−	−	−	−	−	−	−	−	
S 267	S 60	Type S 3	−	−	−	−	−	−	−	−	−	−
		Normal.	−	−	−	−	−	−	−	−	−	
S 4	S 60	Type S 3	−	−	−	−	−	−	−	−	−	−
		Normal.	−	−	−	−	−	−	−	−	−	
S 84	S 84	Type S 3	−	−	−	−	−	−	−	−	−	−
		Normal.	−	−	−	−	−	−	−	−	−	
S 1	S 84	Type S 3	−	−	−	−	−	−	−	−	−	−
		Normal.	−	−	−	−	−	−	−	−	−	
S 50	S 84	Type S 3	−	−	−	−	−	−	−	−	−	−
		Normal.	−	−	−	−	−	−	−	−	−	
S 20	S 84	Type S 3	−	−	−	−	−	−	−	−	−	−
		Normal.	−	−	−	−	−	−	−	−	−	
S 115	S 84	Type S 3	−	−	−	−	−	−	−	−	−	−
		Normal.	−	−	−	−	−	−	−	−	−	
S 31	Unclassified.	Type S 3	+	+	+	+	+					−
		Normal.	−	−	−	−	−					
S 108	"	Type S 3	−	−	−	−	−	−	−			−
		Normal.	−	−	−	−	−	−	−			
S 87	"	Type S 3	−	−	−	−	−	−	−			−
		Normal.	−	−	−	−	−	−	−			
S 288	"	Type S 3	−	−	−	−	−	−	−			−
		Normal.	−	−	−	−	−	−	−			
S 42	"	Type S 3	−	−	−	−	−	−	−			−
		Normal.	−	−	−	−	−	−	−			
S 121	"	Type S 3	+	±	±	−	−	−	−			−
		Normal.	−	−	−	−	−	−	−			

allowed to continue longer, non-specific granulation occurs. If clumping develops in the broth controls or in more than the first two or three dilutions of normal serum, the reaction should be regarded as unsatisfactory and discarded. By the use of this technique, it has been possible to carry out reliable agglutination tests of various strains of *Streptococcus hæmolyticus* and to show that constant type rela-

TABLE IV.

Power of Antistreptococcus Serum, Type S 23, to Agglutinate Ten Representative Strains of the Same Type.

Strain No.	Serum.	Dilution.									
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	Broth.
S 23	Type S 23	++	++	++	++	++	++	+	−	−	−
	Normal.	−	−	−	−	−	−	−	−	−	
S 107	Type S 23	++	++	++	++	++	++	+±	+	±	−
	Normal.	−	−	−	−	−	−	−	−	−	
S 27	Type S 23	+	++	++	++	+±	+	±	−	−	−
	Normal.	−	−	−	−	−	−	−	−	−	
S 39	Type S 23	+	+	+±	++	+±	+	±	−	−	−
	Normal.	−	−	−	−	−	−	−	−	−	
S 56	Type S 23	+	+	+±	+±	+±	+	±	−	−	−
	Normal.	−	−	−	−	−	−	−	−	−	
S 67	Type S 23	+	+	+±	+±	+	+	±	−	−	−
	Normal.	−	−	−	−	−	−	−	−	−	
S 98	Type S 23	+±	++	++	++	++	+±	+±	+	−	−
	Normal.	−	−	−	−	−	−	−	−	−	
S 101	Type S 23	+±	++	++	++	++	+±	+±	+	±	−
	Normal.	−	−	−	−	−	−	−	−	−	
S 104	Type S 23	+	++	++	++	++	++	+±	+	−	−
	Normal.	−	−	−	−	−	−	−	−	−	
S 130	Type S 23	+±	+±	+±	++	++	+±	+	+	+	−
	Normal.	−	−	−	−	−	−	−	−	−	

tionships exist, and that the types are sharply differentiated from one another. The results of the application of the method to strains of streptococcus described above are shown in Tables II to IX.

In these tables are presented the agglutination reactions of a certain proportion of the total number of strains of *Streptococcus hæmolyticus* tested. An analysis of the results shows that in the collection of

TABLE V.

Test of Cross-Agglutination Reactions of Antistreptococcus Serum, Type S 23, with Strains of Streptococcus hæmolyticus of Other Types and with Unclassified Strains.

Strain No.	Type of *S. hæmolyticus*.	Serum.	Dilution.									
			1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	Broth.
S 23	S 23	Type S 23	±	+	+±	+±	+±	+±	+±	+	–	–
		Normal.	–	–	–	–	–	–	–	–	–	
S 95	S 3	Type S 23	–	–	–	–	–	–	–	–	–	–
		Normal.	–	–	–	–	–	–	–	–	–	
S 149	S 3	Type S 23	–	–	–	–	–	–	–	–	–	–
		Normal.	–	–	–	–	–	–	–	–	–	
S 80	S 3	Type S 23	–	–	–	–	–	–	–	–	–	–
		Normal.	–	–	–	–	–	–	–	–	–	
S 146	S 3	Type S 23	–	–	–	–	–	–	–	–	–	–
		Normal.	–	–	–	–	–	–	–	–	–	
S 125	S 3	Type S 23	–	–	–	–	–	–	–	–	–	–
		Normal.	–	–	–	–	–	–	–	–	–	
S 55	S 60	Type S 23	–	–	–	–	–	–	–	–	–	–
		Normal.	–	–	–	–	–	–	–	–	–	
S 60	S 60	Type S 23	–	–	–	–	–	–	–	–	–	–
		Normal.	–	–	–	–	–	–	–	–	–	
S 4	S 60	Type S 23	–	–	–	–	–	–	–	–	–	–
		Normal.	–	–	–	–	–	–	–	–	–	
S 19	S 60	Type S 23	–	–	–	–	–	–	–	–	–	–
		Normal.	–	–	–	–	–	–	–	–	–	
S 6	S 60	Type S 23	–	–	–	–	–	–	–	–	–	–
		Normal.	–	–	–	–	–	–	–	–	–	
S 20	S 84	Type S 23	–	–	–	–	–	–	–	–	–	–
		Normal.	–	–	–	–	–	–	–	–	–	
S 50	S 84	Type S 23	–	–	–	–	–	–	–	–	–	–
		Normal.	–	–	–	–	–	–	–	–	–	
S 84	S 84	Type S 23	–	–	–	–	–	–	–	–	–	–
		Normal.	–	–	–	–	–	–	–	–	–	
S 115	S 84	Type S 23	–	–	–	–	–	–	–	–	–	–
		Normal.	–	–	–	–	–	–	–	–	–	
S 1	S 84	Type S 23	–	–	–	–	–	–	–	–	–	–
		Normal.	–	–	–	–	–	–	–	–	–	
S 15	Unclassified.	Type S 23	–	–	–	–	–	–	–	–	–	–
		Normal.	–	–	–	–	–	–	–	–	–	
S 116	"	Type S 23	–	–	–	–	–	–	–	–	–	–
		Normal.	–	–	–	–	–	–	–	–	–	
S 49	"	Type S 23	–	–	–	–	–	–	–	–	–	–
		Normal.	–	–	–	–	–	–	–	–	–	
S 69	"	Type S 23	–	–	–	–	–	–	–	–	–	–
		Normal.	–	–	–	–	–	–	–	–	–	
S 155	"	Type S 23	–	–	–	–	–	–	–	–	–	–
		Normal.	–	–	–	–	–	–	–	–	–	
S 18	"	Type S 23	–	–	–	–	–	–	–	–	–	–
		Normal.	–	–	–	–	–	–	–	–	–	
S 99	"	Type S 23	–	–	–	–	–	–	–	–	–	–
		Normal.	–	–	–	–	–	–	–	–	–	
S 24	"	Type S 23	–	–	–	–	–	–	–	–	–	–
		Normal.	–	–	–	–	–	–	–	–	–	

organisms studied it has been possible to detect four different types of *Streptococcus hæmolyticus*. These types have been noted as Types S 3, S 23, S 60, and S 84, from the number of the chosen representative. In addition to the type strains, there remains a residue of unclassified organisms. The summary for the total number of strains studied is given in Table X.

TABLE VI.

Power of Antistreptococcus Serum, Type S 60, to Agglutinate Ten Representative Strains of the Same Type.

Strain No.	Serum.	Dilution.									
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	Broth.
S 60	Type S 60	++	++	++		++		+±	+±	+	−
	Normal.	−	−	−		−		−	−	−	
S 269	Type S 60	+±	+±	+±	+±	+	+	±	−		−
	Normal.	±	−	−	−	−	−	−	−		
S 267	Type S 60	++	++	++	++	+±	+±	+	+		−
	Normal.	±	−	−	−	−	−	−	−		
S 55	Type S 60	++	++	++		+±		±	±	±	−
	Normal.	−	−	−		−		−	−	−	
S 128	Type S 60	++	++	++		++		+±	+±	+	−
	Normal.	−	−	−		−		−	−	−	
S 72	Type S 60	+±	+±		+±		+±	+	+	±	−
	Normal.	±	±		−		−	−	−	−	
S 43	Type S 60	++	++		+±		+±	+±	+±	+	−
	Normal.	−	−		−		−	−	−	−	
S 123	Type S 60	++	++		++		++	+±	+±	+	−
	Normal.	−	−		−		−	−	−	−	
S 66	Type S 60	++	++		++		+±	+±	+	+	−
	Normal.	−	−		−		−	−	−	−	
S 127	Type S 60	++	++		++		++	+±	+±	+	−
	Normal.	−	−		−		−	−	−	−	

The total number of strains of *Streptococcus hæmolyticus* studied was 125. Of these, 85, or 68 per cent, are comprised in the types mentioned above, and 40, or 32 per cent, remain unclassified. Work with the unclassified strains is being continued and the indications are that a certain number of other types will be discovered. In fact, two new types have already been encountered, one comprising five

TABLE VII.

Test of Cross-Agglutination Reactions of Antistreptococcus Serum, Type S 60, with Strains of Streptococcus hæmolyticus of Other Types and with Unclassified Strains.

Strain No.	Type of *S. hæmolyticus.*	Serum.	Dilution.									
			1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	Broth.
S 60	S 60	Type S 60	++	++	++	+±	+±	+	±	±		−
		Normal.	−	−	−	−	−	−	−	−		
S 3	S 3	Type S 60	−	−	−	−	−	−	−	−		−
		Normal.	−	−	−	−	−	−	−	−		
S 111	S 3	Type S 60	−	−	−	−	−	−	−	−		−
		Normal.	−	−	−	−	−	−	−	−		
S 146	S 3	Type S 60	−	−	−	−	−	−	−	−		−
		Normal.	−	−	−	−	−	−	−			
S 80	S 3	Type S 60	−	−	−	−	−	−	−			−
		Normal.	−	−	−	−	−	−	−			
S 14	S 3	Type S 60	−	−	−	−	−	−	−			−
		Normal.	−	−	−	−	−	−	−			
S 23	S 23	Type S 60	±	−	−	−	−	−	−			−
		Normal.	±	±	−	−	−	−	−			
S 78	S 23	Type S 60	−	−	−	−	−	−	−	−		−
		Normal.	−	−	−	−	−	−	−	−		
S 98	S 23	Type S 60	−	−	−	−	−	−	−	−		−
		Normal.	−	−	−	−	−	−	−	−		
S 107	S 23	Type S 60	−	−	−	−	−	−	−			−
		Normal.	−	−	−	−	−	−	−			
S 122	S 23	Type S 60	−	−	−	−	−	−	−			−
		Normal.	−	−	−	−	−	−	−			
S 84	S 84	Type S 60	−	−	−	−	−	−	−			−
		Normal.	−	−	−	−	−	−	−			
S 115	S 84	Type S 60	−	−	−	−	−	−	−	−		−
		Normal.	−	−	−	−	−	−	−	−		
S 15	S 84	Type S 60	±	±	−	−	−	−	−			−
		Normal.	−	−	−	−	−	−	−			
S 1	S 84	Type S 60	−	−	−	−	−	−	−			−
		Normal.	−	−	−	−	−	−	−			
S 20	S 84	Type S 60	−	−	−	−	−	−	−			−
		Normal.	−	−	−	−	−	−	−			
S 148	Unclassified.	Type S 60	+	−	−	−	−	−	−	−		−
		Normal.	−	−	−	−	−	−	−	−		
S 96	"	Type S 60	+	±	±	−	−	−	−	−		−
		Normal.	−	−	−	−	−	−	−	−		
S 286	"	Type S 60	±	±		±		−	−	−		−
		Normal.	−	−		−		−	−	−		
S 47	"	Type S 60	+±	+	+	−	−	−	−	−		−
		Normal.	−	−	−	−	−	−	−	−		
S 102	"	Type S 60	−	+	+	+	±	±	−	−		−
		Normal.	−	−	−	−	−	−	−	−		
S 48	"	Type S 60	±	−	−		−		−	−	−	−
		Normal.	−	−	−		−		−	−	−	
S 17	"	Type S 60	−	−		−		−	−	−	−	−
		Normal.	−	−		−		−	−	−	−	

strains and another four strains, the immune reactions of which have not as yet been completed.

The antistreptococcus sera used in the agglutination reaction were obtained in the main by the immunization of sheep, a species of animal which yields a highly specific agglutinating serum for *Streptococcus hæmolyticus*. Agglutination occurred in all the type sera in dilutions of 1 : 1,000 or higher, with the exception of Type S 84, of which the

TABLE VIII.

Power of Antistreptococcus Serum, Type S 84, to Agglutinate Eight Representative Strains of the Same Type.

Strain No.	Serum.	Dilution.									
		1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	Broth.
S 84	Type S 84	+±		+±	+	+	±				−
	Normal.	−		−	−	−	−				
S 1	Type S 84	+±	+±	+±	+±	+±	±				−
	Normal.	−	−	−	−	−	−				
S 50	Type S 84	+±	+±	+±	+±	+±	+				−
	Normal.	−	−	−	−	−	−				
S 20	Type S 84	++	++	+±	+±	±	−				−
	Normal.	−	−	−	−	−	−				
S 15	Type S 84	+	+±	+±	+±	+					−
	Normal.	−	−	−	−	−					
S 115	Type S 84	+±	+±	+±	+±	±					−
	Normal.	−	−	−	−	−					
S 139	Type S 84	+±	+±	+±	+±	+					−
	Normal.	−	−	−	−	−					
S 138	Type S 84	+±	+±	+±	+±	+					−
	Normal.	−	−	−	−	−					

agglutination titer has been consistently lower, usually not above 1:320. The agglutination titer of all the type sera for each strain of the same type has been approximately equal to the titer for the organism used for purposes of immunization. There has been strikingly little cross-agglutination between serum of one type and strains belonging to another. The same lack of crossing is observed among the unclassified strains with the few exceptions in which certain of these strains have shown some degree of agglutination in the type

TABLE IX.

Test of Cross-Agglutination Reactions of Antistreptococcus Serum, Type S 84, with Strains of Streptococcus hæmolyticus of Other Types and with Unclassified Strains.

Strain No.	Type of *S. hæmolyticus.*	Serum.	Dilution.									
			1:20	1:40	1:80	1:160	1:320	1:640	1:1,280	1:2,560	1:5,120	Broth.
S 84	S 84	Type S 84	+±	+±	+±	+	+	+	+			−
		Normal.	−	−	−	−	−	−	−			
S 3	S 3	Type S 84	−	−	−	−	−	−	−			−
		Normal.	−	−	−	−	−	−	−			
S 14	S 3	Type S 84	−	−	−	−	−	−	−			−
		Normal.	−	−	−	−	−	−	−			
S 80	S 3	Type S 84	−	−	−	−	−	−	−			−
		Normal.	−	−	−	−	−	−	−			
S 146	S 3	Type S 84	−	−	−	−	−	−	−			−
		Normal.	−	−	−	−	−	−	−			
S 95	S 3	Type S 84	±	±	−	−	−	−	−			−
		Normal.	−	−	−	−	−	−	−			
S 23	S 23	Type S 84	±	±	−	−	−	−	−			−
		Normal.	±	±	−	−	−	−	−			
S 107	S 23	Type S 84	±	±	−	−	−	−	−			−
		Normal.	−	−	−	−	−	−	−			
S 122	S 23	Type S 84	−	−	−	−	−	−	−			−
		Normal.	−	−	−	−	−	−	−			
S 78	S 23	Type S 84	−	−	−	−	−	−	−			−
		Normal.	−	−	−	−	−	−	−			
S 98	S 23	Type S 84	±	−	−	−	−	−	−			−
		Normal.	±	±	−	−	−	−	−			
S 60	S 60	Type S 84	+	+	±	±	−	−	−			−
		Normal.	±	±	±	±	−	−	−			
S 128	S 60	Type S 84	+±	+	+	+	−	−	−			−
		Normal.	+	+	±	±	−	−	−			
S 19	S 60	Type S 84	+	±	±	−	−	−	−			−
		Normal.	±	±	−	−	−	−	−			
S 141	S 60	Type S 84	+	±	±	−	−	−	−			−
		Normal.	±	±	±	−	−	−	−			
S 4	S 60	Type S 84	+	±	±	−	−	−	−			−
		Normal.	+	±	±	−	−	−	−			
S 108	Unclassified.	Type S 84	−	−	−	−	−					−
		Normal.	−	−	−	−	−					
S 97	"	Type S 84	−	−	−	−	−					−
		Normal.	−	−	−	−	−					
S 54	"	Type S 84	−	−	−	−	−					−
		Normal.	−	−	−	−	−					
S 34	"	Type S 84	−	−	−	−	−					−
		Normal.	−	−	−	−	−					
S 106	"	Type S 84	−	−	−	±	±					−
		Normal.	−	−	−	−	−					

sera, but not in sufficiently high dilutions to justify their inclusion within the types. These facts show that it is possible by a series of carefully conducted agglutination experiments to determine specific type relationships between strains of *Streptococcus hæmolyticus* and to show that the different types are immunologically distinct from one another. The clearness of the agglutination reactions presented is somewhat deceptive as to the ease and simplicity of the test. It must be remembered that *Streptococcus hæmolyticus* is notoriously

TABLE X.

Summary of Agglutination Reactions.

Type of *S. hæmolyticus*.	No. of strains.	Per cent.
S 3	29	23.2
S 23	20	16.0
S 60	27	21.6
S 84	9	7.2
Unclassified	40	32.0
Total typed	85	68.0
" strains studied	125	

variable in its reactions, and that very slight and indeterminable changes in technique frequently obliterate almost completely the specificity of the agglutination reaction. In addition, a considerable number of strains is invincibly granular under all conditions and cannot be used, and occasionally strains are encountered which may occupy intermediate positions, the exact understanding of which needs a technique for the conduction of absorption experiments.

The Reaction of Protection.

Study of the power of antistreptococcus serum to protect animals against experimental infection with this organism has given rise to a number of different points of view, both regarding its action against strains from different sources, and against the same strain before and after animal passage, and also concerning the kind and the different

effect of varying antigens used in the process of immunization. For a full discussion of these matters the reader is referred to the general articles on streptococcus and to the more important papers dealing with these particular points (6). In this work they will only be considered where they have a particular bearing upon the subject under investigation. Although in the present paper the classification of *Streptococcus hæmolyticus* by means of the agglutination reaction has been presented first, practically we have obtained our primary indications of the degree of antigenic differences between strains by means of the reaction of protection. Later each reaction has been used to confirm the information obtained by means of the other.

In the successful carrying out of protection experiments two points are of especial importance: first, the production of a serum of high potency; and second, the possibility of raising the virulence of the test strains of streptococcus to such a point that very minute doses of culture are sufficient to kill white mice in a limited period of time. We have been able to produce sera in the manner alluded to above of such potency that 0.5 cc. administered intraperitoneally is sufficient to protect a white mouse against 100,000 lethal doses of a highly virulent streptococcus. In order to produce such a serum many animals must be used, only a few of which may give the desired result. It has been possible to raise the virulence of many strains by continuous passage through white mice and rats to such a point that doses of from 0.000001 to 0.00000001 cc. of broth culture are sufficient to kill the former animals in from 24 to 48 hours. These seemingly difficult conditions must be attained in order that sufficiently long-range protective titers may be carried out to insure the reliability of the information obtained. Protection against one or two lethal doses of a series of strains of streptococcus by a monovalent serum is subject to so many interpretations that the evidence gained cannot be considered of much value in judging accurately the antigenic relationship of the different strains.

The technique observed in the protocols given below has been as follows: The potency of all sera has been titrated for the homologous strain of organism and only the sera which gave a wide range of protection have been used. For infection, virulent streptococci have been used which have been grown for approximately 18 hours in either

plain broth or ascites broth. In the inoculation of animals the technique advised by Neufeld (5) has been followed with only a minor variation. The test animals have been injected intraperitoneally with 0.5 cc. of serum 24 hours before the conduction of the experiment. Tentative trials have shown that if the serum is given simultaneously with the infecting dose, no protection results, and that to insure success the serum must be given at least 8 hours before infection. On the following day a series of virulence controls is inoculated intraperitoneally, and the serum animals are injected in the same manner with doses of cultures ranging from 0.001 to 0.00000001 cc. of broth culture. Animals surviving for a period of 5 days are considered to be adequately protected. By the use of this method, it has been possible to test the antigenic relationship of a considerable number of virulent strains of *Streptococcus hæmolyticus*, and the results of these tests are set forth in the following protocols.

Protocol 1.

In this protocol is shown the titration of the serum of a sheep immunized against Strain S 23. The culture employed for infection was an 18 hour broth culture of No. S 23, which had received eighteen passages through white rats and mice. Each mouse had received 0.5 cc. of immune serum 24 hours previous to infection.

Virulence controls.		Protective power of Serum S 23.	
Dose of culture.	Result.	Dose of culture.	Result.
cc.		*cc.*	
		0.001	S.
		0.0001	D. in 4 days.
0.00001	D.* in 24 hrs.	0.000001	S.
0.000001	" "24 "	0.0000001	"
0.0000001	" "24 "	0.0000001	"

* In the tables D. indicates died, S. survived.

Protocol 2.

In this protocol is shown the protective power of Immune Serum S 3 for two virulent strains of the homologous type, for two strains of each of the heterologous types, and for two unclassified strains. The technique was the same as that in the previous protocol.

Streptococcus No.	Type of streptococcus.	Type of serum.	Dose of culture.					
			0.001 cc.	0.0001 cc.	0.00001 cc.	0.000001 cc.	0.0000001 cc.	0.00000001 cc.
S 3.18[2]*	S 3	S 3	D. 14 hrs.	S.	S.	S.	S.	
		No serum.				D. 20 hrs.	D. 23 hrs.	
S 149.16[2]	S 3	S 3	S.	S.	S.	S.		
		No serum.		D. 24 hrs.	D. 36 hrs.	D. 24 hrs.		
S 39.3[2]	S 23	S 3	D. 16 hrs.	" 16 "	" 7 "	" 19 "		
		No serum.		" 16 "	" 16 "	" 21 "		
S 67.7[2]	S 23	S 3	D. 8 hrs.	" 8 "	" 17 "	" 17 "	D. 19 hrs.	
		No serum.				" 23 "	" 21 "	
S 128.14[2]	S 60	S 3	D. 24 hrs.	D. 24 hrs.	D. 48 hrs.	" 24 "		
		No serum.		" 24 "	" 36 "	" 36 "		
S 60.10[2]	S 60	S 3	D. 18 hrs.	" 36 "	" 36 "	" 20 "		
		No serum.		" 18 "	S.	" 24 "		
S 1.8[1]	S 84	S 3	D. 22 hrs.	" 22 "	D. 22 hrs.	" 22 "		
		No serum.		" 21 "	" 21 "	" 21 "		
S 84.17[2]	S 84	S 3		" 13 "	" 19 "	" 19 "	S.	D. 20 hrs.
		No serum.				" 18 "	D. 21 hrs.	" 60 "
S 24.25[3]	Unclassified.	S 3	D. 14 hrs.	D. 16 hrs.	D. 19 hrs.	" 31 "	" 19 "	
		No serum.				" 21 "	" 28 "	
S 266.6[2]	"	S 3	D. 16 hrs.	D. 16 hrs.	D. 19 hrs.	" 21 "		
		No serum.		" 17 "	" 21 "	" 24 "		

* The integer indicates serial number of the culture, the decimal shows the number of animal passages, and the exponent the number of transplants since the last animal passage.

Protocol 3.

In this protocol is shown the protective power of antistreptococcus serum, Type S 23, against two strains of the homologous type, against two strains of each of the heterologous types, and against two unclassified strains. The technique was the same as that employed in the previous protocols.

Streptococcus No.	Type of streptococcus.	Type of serum.	Dose of culture.				
			0.001 cc.	0.0001 cc.	0.00001 cc.	0.000001 cc.	0.0000001 cc.
S 23.18[2]	S 23	S 23	D. 11 hrs.	S.	S.	S.	
		No serum.			D. 19 hrs.	D. 24 hrs.	D. 20 hrs.
S 107.12[2]	S 23	S 23	S.	S.	" 16 "	S.	S.
		No serum.			" 24 "	D. 24 hrs.	D. 24 hrs.
S 3.21[2]	S 3	S 23	D. 18 hrs.	D. 18 hrs.	" 18 "	" 36 "	" 64 "
		No serum.			" 18 "	" 36 "	" 36 "
S 80.7[2]	S 3	S 23	D. 18 hrs.	D. 18 hrs.	" 64 "	" 96 "	S.
		No serum.			S.	" 36 "	D. 36 hrs.
S 55.22[1]	S 60	S 23	D. 20 hrs.	D. 36 hrs.	D. 20 hrs.	" 30 "	
		No serum.		" 21 "	" 22 "	" 20 "	
S 60.10[1]	S 60	S 23	D. 20 hrs.	" 20 "	" 24 "	S.	
		No serum.		" 20 "	" 20 "	D. 36 hrs.	
S 84.18[1]	S 84	S 23	D. 12 hrs.	" 20 "	" 18 "	" 36 "	D. 18 hrs.
		No serum.			" 16 "	" 20 "	S.
S 50.4[2]	S 84	S 23	D. 18 hrs.	D. 36 hrs.	" 18 "	" 18 "	D. 18 hrs.
		No serum.			" 18 "	" 36 "	" 18 "
S 276.31[2]	Unclassified.	S 23	D. 16 hrs.	D. 40 hrs.	" 66 "	" 40 "	
		No serum.		" 20 "	" 18 "	" 40 "	
S 24.31[2]	"	S 23	D. 16 hrs.	" 16 "	" 16 "		
		No serum.			" 16 "	D. 20 hrs.	

Protocol 4.

In this protocol is shown the protective power of antistreptococcus serum, Type S 60, against two strains of homologous type, against two strains of each of the heterologous types, and against two unclassified strains. The technique was the same as that employed in the previous protocols.

Streptococcus No.	Type of streptococcus.	Type of serum.	Dose of culture.				
			0.001 cc.	0.0001 cc.	0.00001 cc.	0.000001 cc.	0.0000001 cc.
S 60.10[2]	S 60	S 60	D. 19 hrs.	S.	S.	S.	
		No serum.			D. 17 hrs.	D. 19 hrs.	
S 55.22[2]	S 60	S 60	S.	S.	S.	S.	
		No serum.			D. 23 hrs.	D. 16 hrs.	
S 3.22[2]	S 3	S 60	D. 18 hrs.	D. 18 hrs.	" 18 "	" 30 "	D. 24 hrs.
		No serum.			" 18 "	" 18 "	" 18 "
S 80.8[2]	S 3	S 60	D. 18 hrs.	D. 18 hrs.	" 18 "	" 22 "	" 24 "
		No serum.			" 18 "	" 18 "	" 18 "
S 75.6[1]	S 23	S 60	D. 18 hrs.	D. 21 hrs.	" 20 "		
		No serum.		" 23 "	" 17 "	D. 23 hrs.	
S 23.19[2]	S 23	S 60	D. 18 hrs.	" 18 "	" 18 "	" 24 "	D. 60 hrs.
		No serum.			" 18 "	" 60 "	" 18 "
S 84.12[1]	S 84	S 60	D. 18 hrs.	D. 18 hrs.	" 18 "	" 22 "	
		No serum.		" 18 "	" 18 "	" 20 "	
S 50.5[2]	S 84	S 60	D. 18 hrs.	" 18 "	" 18 "	" 18 "	D. 18 hrs.
		No serum.			" 18 "	" 18 "	" 18 "
S 24.28[2]	Unclassified.	S 60	D. 17 hrs.	D. 17 hrs.	" 17 "	" 24 "	
		No serum.			" 17 "	" 65 "	D. 31 hrs.
S 276.31[2]	"	S 60	D. 9 hrs.	D. 20 hrs.	" 24 "	" 41 "	
		No serum.		" 23 "	" 48 "	" 33 "	

Protocol 5.

In this protocol is shown the protective power of antistreptococcus serum, Type S 84, against two strains of the homologous type, against two strains of each of the heterologous types, and against two unclassified strains. The technique was the same as that employed in previous protocols.

Streptococcus No.	Type of streptococcus.	Type of serum.	Dose of culture.				
			0.001 cc.	0.0001 cc.	0.00001 cc.	0.000001 cc.	0.0000001 cc.
S 84.16[1]	S 84	S 84	S.	S.	S.	S.	
		No serum.		D. 16 hrs.	D. 26 hrs.	D. 54 hrs.	
S 20.5[1]	S 84	S 84	S.	S.	S.	S.	
		No serum.		D. 30 hrs.	D. 55 hrs.	D. 55 hrs.	
S 3.16[1]	S 3	S 84	D. 17 hrs.	" 17 "	" 20 "	" 29 "	
		No serum.		" 17 "	" 20 "	" 29 "	
S 14.24[1]	S 3	S 84	D. 16 hrs.	" 16 "	" 16 "	" 16 "	D. 16 hrs.
		No serum.				" 16 "	" 40 "
S 23.16[1]	S 23	S 84	D. 18 hrs.	D. 18 hrs.	D. 33 hrs.	" 32 "	
		No serum.		S.	" 22 "	" 23 "	
S 107.7[1]	S 23	S 84	D. 18 hrs.	D. 18 hrs.	" 24 "	S.	
		No serum.			" 18 "	D. 18 hrs.	D. 24 hrs.
S 128.14[2]	S 60	S 84	D. 24 hrs.	D. 24 hrs.	" 24 "	" 24 "	
		No serum.			" 24 "	" 36 "	
S 60.10[2]	S 60	S 84	D. 18 hrs.	D. 18 hrs.	" 18 "	" 21 "	
		No serum.		" 18 "	S.	" 24 "	
S 152.5[1]	Unclassified.	S 84	D. 20 hrs.	" 29 "	D. 29 hrs.	" 76 "	
		No serum.		" 16 "	S.	" 28 "	
S 266.5[2]	"	S 84	D. 15 hrs.	" 34 "	D. 23 hrs.	" 45 "	
		No serum.		" 18 "	" 38 "	" 21 "	

Consideration of the above protocols shows that the type relationships manifest from the agglutination reactions have been substantiated by the evidence obtained from the protection tests. As a matter of fact, each reaction has been used to supplement the other, the first clue as to the position of an organism sometimes being obtained by protection and sometimes by agglutination. On the whole, we are inclined to place greater confidence in the reaction of protection than in that of agglutination, and would be slow to draw general conclusions concerning type specificity from agglutination alone with such a variable organism as streptococcus, unless the results of this test could be confirmed by some other specific reaction such as protection. The sera prepared, as is seen from the protocols, have afforded a high degree of protection to white mice against infection with organisms of the homologous type. Little or no protection results when serum of one type is employed against organisms of heterologous types. There are, of course, some exceptions to this general rule. Occasionally strains of *Streptococcus hæmolyticus* are encountered against which all type sera afford a varying degree of protection, and sometimes a serum is obtained from one strain which will protect against an organism of another type, and when the reaction is reciprocally reversed no protection results. At present our knowledge is insufficient to discuss these intermediate reactions intelligently, and their elucidation must await further development of the technique. In all it has been possible to raise the virulence of 31 strains to a point where protection experiments could be performed. Of these, 7 belonged to Type S 3, 8 to Type S 23, 6 to Type S 60, 7 to Type S 84, and 3 to the unclassified group. In view of the difficulty of raising the virulence of the organisms it has been found advantageous to perform the reaction in two ways: first, to test a single monovalent serum against a number of strains; and second, to test a number of sera prepared from strains of the same type against a single virulent strain of that type. In Tables XI to XIV is shown a summary of the total number of protection experiments performed.

TABLE XI.

Summary of the Protective Power of Antistreptococcus Serum, Type S 3, against Strains of the Homologous and Heterologous Types.

Type of serum.	Strain of *S. hæmolyticus* used for production of serum.	Strain of *S. hæmolyticus* and type used for infection of mice.	Minimal lethal dose of *S. hæmolyticus* used for infection.	Protective power of 0.5 cc. of serum.
			cc.	
S 3	S 3 (Rabbit 1).	S 3 (Type S 3).	0.00000001	S. 0.0001 cc.
S 3	S 3 (" 1).	S 14 (" S 3).	0.000001	" 0.001 "
S 3	S 3 (Dog 1).	S 3 (" S 3).	0.00000001	" 0.001 "
S 3	S 3 (" 1).	S 95 (" S 3).	0.00001	" 0.01 "
S 3	S 3 (" 1).	S 80 (" S 3).	0.000001	" 0.001 "
S 3	S 3 (" 1).	S 149 (" S 3).	0.000001	" 0.01 "
S 3	S 3 (" 1).	S 146 (" S 3).	0.000001	" 0.01 "
S 3	S 3 (" 1).	S 144 (" S 3).	0.00001	" 0.001 "
S 3	S 111 (Rabbit 2).	S 3 (" S 3).	0.0000001	" 0.001 "
S 3	S 118 (" 3).	S 3 (" S 3).	0.0000001	" 0.0001 "
S 3	S 2 (" 4).	S 3 (" S 3).	0.0000001	" 0.001 "
S 3	S 11 (" 5).	S 3 (" S 3).	0.0000001	" 0.001 "
S 3	S 29 (" 6).	S 3 (" S 3).	0.00000001	" 0.0001 "
S 3	S 16 (" 7).	S 3 (" S 3).	0.00000001	" 0.0001 "
S 3	S 3 (" 1).	S 107 (" S 23).	0.0000001	D. 0.0000001 "
S 3	S 3 (" 1).	S 23 (" S 23).	0.0000001	" 0.0000001 "
S 3	S 3 (Dog 1).	S 27 (" S 23).	0.000001	" 0.000001 "
S 3	S 3 (" 1).	S 67 (" S 23).	0.00000001	" 0.0000001 "
S 3	S 3 (" 1).	S 39 (" S 23).	0.000001	" 0.000001 "
S 3	S 3 (" 1).	S 75 (" S 23).	0.000001	" 0.00001 "
S 3	S 3 (" 2).	S 56 (" S 23).	0.000001	" 0.000001 "
S 3	S 3 (" 1).	S 128 (" S 60).	0.000001	" 0.000001 "
S 3	S 3 (" 1).	S 60 (" S 60).	0.000001	" 0.000001 "
S 3	S 3 (Rabbit 1).	S 84 (" S 84).	0.00000001	" 0.00000001 "
S 3	S 3 (" 1).	S 1 (" S 84).	0.000001	" 0.000001 "
S 3	S 3 (" 1).	S 24 (unclassified).	0.00000001	" 0.00000001 "
S 3	S 3 (" 1).	S 276 (").	0.0000001	S. 0.0000001 "
S 3	S 3 (" 1).	S 61 (").	0.00001	" 0.00001 "
S 3	S 3 (Dog 1).	S 152 (").	0.000001	D. 0.000001 "
S 3	S 3 (" 1).	S 266 (").	0.000001	" 0.000001 "
Unclassified.	S 24 (Rabbit 8).	S 3 (Type S 3).	0.000001	" 0.000001 "
"	S 24 (Sheep 2).	S 14 (" S 3).	0.00000001	S. 0.00001 "
"	S 276 (Rabbit 9).	S 14 (" S 3).	0.000001	" 0.000001 "

TABLE XII.

Summary of the Protective Power of Antistreptococcus Serum, Type S 23, against Strains of the Homologous and Heterologous Types.

Type of serum.	Strain of *S. hæmolyticus* used for production of serum.	Strain of *S. hæmolyticus* and type used for infection of mice.	Minimal lethal dose of *S. hæmolyticus* used for infection.	Protective power of 0.5 cc. of serum.
			cc.	
S 23	S 23 (Sheep 1).	S 23 (Type S 23).	0.0000001	S. 0.001 cc.
S 23	S 23 (" 1).	S 107 (" S 23).	0.0000001	" 0.001 "
S 23	S 23 (" 1).	S 27 (" S 23).	0.000001	" 0.0001 "
S 23	S 23 (" 1).	S 75 (" S 23).	0.0000001	" 0.001 "
S 23	S 23 (" 1).	S 65 (" S 23).	0.0000001	" 0.001 "
S 23	S 23 (" 1).	S 3 (" S 3).	0.0000001	D. 0.000001 "
S 23.	S 23 (" 1).	S 80 (" S 3).	0.0000001	S. 0.0000001 "
S 23	S 23 (" 1).	S 55 (" S 60).	0.000001	D. 0.000001 "
S 23	S 23 (" 1).	S 60 (" S 60).	0.000001	S. 0.000001 "
S 23	S 23 (" 1).	S 128 (" S 60).	0.000001	D. 0.000001 "
S 23	S 23 (" 1).	S 50 (" S 84).	0.0000001	" 0.0000001 "
S 23	S 23 (" 1).	S 84 (" S 84).	0.000001	" 0.0000001 "
S 23	S 23 (" 1).	S 24 (unclassified).	0.000001	" 0.00001 "
S 23	S 23 (" 1).	S 276 (").	0.000001	" 0.000001 "
Unclassified.	S 24 (" 2).	S 27 (Type S 23).	0.000001	" 0.000001 "
"	S 24 (" 2).	S 56 (" S 23).	0.000001	" 0.000001 "
"	S 24 (" 2).	S 107 (" S 23).	0.000001	" 0.000001 "
"	S 24 (" 2).	S 39 (" S 23).	0.00000001	" 0.000001 "
"	S 24 (" 2).	S 23 (" S 23).	0.00000001	" 0.0000001 "
"	S 276 (Rabbit 9).	S 27 (" S 23).	0.000001	" 0.000001 "
"	S 276 (" 9).	S 56 (" S 23).	0.00001	" 0.000001 "
"	S 276 (" 9).	S 107 (" S 23).	0.0000001	" 0.00000001 "
"	S 276 (" 9).	S 39 (" S 23).	0.000001	" 0.000001 "
"	S 276 (" 9).	S 23 (" S 23).	0.00000001	" 0.00000001 "
"	S 276 (" 9).	S 67 (" S 23).	0.000001	" 0.000001 "

TABLE XIII.

Summary of the Protective Power of Antistreptococcus Serum, Type S 60, against Strains of the Homologous and Heterologous Types.

Type of serum.	Strain of *S. hæmolyticus* used for production of serum.	Strain of *S. hæmolyticus* and type used for infection of mice.	Minimal lethal dose of *S. hæmolyticus* used for infection.	Protective power of 0.5 cc. of serum.
			cc.	
S 60	S 128 (Rabbit 10).	S 128 (Type S 60).	0.00001	S. 0.01 cc.
S 60	S 128 (" 10).	S 60 (" S 60).	0.000001	" 0.0001 "
S 60	S 128 (" 10).	S 55 (" S 60).	0.000001	" 0.001 "
S 60	S 128 (" 10).	S 4 (" S 60).	0.000001	" 0.001 "
S 60	S 128 (" 10).	S 72 (" S 60).	0.00001	" 0.001 "
S 60	S 128 (" 10).	S 267 (" S 60).	0.000001	" 0.001 "
S 60	S 128 (" 10).	S 3 (" S 3).	0.0000001	D. 0.0000001 "
S 60	S 128 (" 10).	S 80 (" S 3).	0.0000001	" 0.0000001 "
S 60	S 128 (" 10).	S 75 (" S 23).	0.000001	" 0.00001 "
S 60	S 128 (" 10).	S 65 (" S 23).	0.000001	S. 0.00001 "
S 60	S 128 (" 10).	S 23 (" S 23).	0.0000001	D. 0.0000001 "
S 60	S 128 (" 10).	S 84 (" S 84).	0.000001	" 0.000001 "
S 60	S 128 (" 10).	S 50 (" S 84).	0.0000001	" 0.0000001 "
S 60	S 128 (" 10).	S 24 (unclassified).	0.0000001	" 0.000001 "
S 60	S 128 (" 10).	S 276 (").	0.000001	" 0.000001 "
Unclassified.	S 24 (" 8).	S 128 (Type S 60).	0.000001	S. 0.001 "
"	S 276 (" 9).	S 128 (" S 60).	0.000001	" 0.0001 "

TABLE XIV.

Summary of the Protective Power of Antistreptococcus Serum, Type S 84, against Strains of the Homologous and Heterologous Types.

Type of serum.	Strain of *S. hæmolyticus* used for production of serum.	Strain of *S. hæmolyticus* and type used for infection of mice.	Minimal lethal dose of *S. hæmolyticus* used for infection.	Protective power of 0.5 cc. of serum.
			cc.	
S 84	S 84 (Sheep 3).	S 84 (Type S 84).	0.000001	S. 0.001 cc.
S 84	S 84 (" 3).	S 1 (" S 84).	0.000001	" 0.0005 "
S 84	S 84 (" 3).	S 20 (" S 84).	0.000001	" 0.001 "
S 84	S 84 (" 3).	S 50 (" S 84).	0.000001	" 0.0005 "
S 84	S 84 (" 3).	S 139 (" S 84).	0.000001	" 0.001 "
S 84	S 84 (" 3).	S 110 (" S 84).	0.0000001	" 0.001 "
S 84	S 84 (" 3).	S 15 (" S 84).	0.0000001	" 0.001 "
S 84	S 1 (Rabbit 11).	S 1 (" S 84).	0.00000001	" 0.00001 "
S 84	S 1 (" 11).	S 84 (" S 84).	0.00000001	" 0.0001 "
S 84	S 1 (" 11).	S 20 (" S 84).	0.000001	" 0.001 "
S 84	S 84 (Sheep 3).	S 3 (" S 3).	0.000001	D. 0.000001 "
S 84	S 84 (" 3).	S 14 (" S 3).	0.00000001	S. 0.000001 "
S 84	S 84 (" 3).	S 23 (" S 23).	0.0000001	D. 0.0000001 "
S 84	S 84 (" 3).	S 107 (" S 23).	0.0000001	S. 0.0000001 "
S 84	S 84 (" 3).	S 39 (" S 23).	0.000001	" 0.000001 "
S 84	S 84 (" 3).	S 67 (" S 23).	0.000001	D. 0.000001 "
S 84	S 84 (" 3).	S 27 (" S 23).	0.000001	" 0.000001 "
S 84	S 84 (" 3).	S 56 (" S 23).	0.00001	" 0.000001 "
S 84	S 1 (Rabbit 11).	S 107 (" S 23).	0.0000001	S. 0.00000001 "
S 84	S 1 (" 11).	S 128 (" S 60).	0.000001	D. 0.000001 "
S 84	S 84 (Sheep 3).	S 128 (" S 60).	0.000001	" 0.000001 "
S 84	S 84 (" 3).	S 60 (" S 60).	0.000001	" 0.000001 "
S 84	S 84 (" 3).	S 276 (unclassified).	0.000001	" 0.000001 "
S 84	S 84 (" 3).	S 277 (").	0.000001	S. 0.000001 "
S 84	S 84 (" 3).	S 152 (").	0.000001	D. 0.000001 "
S 84	S 84 (" 3).	S 266 (").	0.000001	" 0.000001 "
S 84	S 84 (" 3).	S 24 (").	0.000001	" 0.000001 "
Unclassified.	S 24 (Rabbit 8).	S 84 (Type S 84).	0.00001	" 0.000001 "
"	S 24 (Sheep 2).	S 1 (" S 84).	0.000001	" 0.000001 "
"	S 276 (Rabbit 9).	S 84 (" S 84).	0.00000001	" 0.00000001 "
"	S 276 (" 9).	S 20 (" S 84).	0.000001	" 0.000001 "
"	S 276 (" 9).	S 50 (" S 84).	0.000001	" 0.000001 "

DISCUSSION.

The complete biological classification of any pathogenic microorganism presents a very complex problem. The first phase of the undertaking concerns itself with the development of reliable methods for the determination of antigenic differences between members of the species and the application of these methods to the discovery of the immunological relationships between a limited number of strains purposefully selected. In this way the degree of similarity and diversity of type is shown and also the probable number of types, and the proportion of classifiable to unclassifiable strains. The next step of necessity is the testing of the adequacy and universality of the information so gained by applying the tentative classification to a large number of strains of the organism obtained under what may be described as normal conditions of pathogenicity. That some sort of equilibrium has been established in nature among microorganisms that have produced disease over long periods of time is not unlikely. Indeed, evidence obtained from the study of pneumococci supports this view (7), although departure from the norm may occur under special conditions (8). After the relationships of the pathogens of the species to one another have been discovered, it then becomes important for purposes of epidemiological study to compare by the same methods the pathogenic with the saprophytic varieties. This task requires years for its completion and many difficulties and seemingly unexplainable phenomena are encountered. In the beginning, the broader lines of differentiation must be drawn, and divergent results discarded for the time being, since, if the original conception is correct, most of the discrepancies disappear with the advance of knowledge.

In this paper are presented the facts so far obtained in the present study of *Streptococcus hæmolyticus* in accordance with the plan outlined above. The strains were collected in a limited community during the course of what may be considered an epidemic of bronchopneumonia secondary to measles. Individuals, however, from all parts of the United States were passing rapidly through this community which was a center for primary training of the aviation service, so that a wider range of territory is represented than the im-

mediate community itself. All the strains were investigated as to their cultural reactions, bile solubility, capacity to hemolyze red blood cells and to ferment the different test sugars, and as to hydrogen ion concentration limiting their growth, and thus identified as accurately as possible as *Streptococcus hæmolyticus* of the human type.

A technique was then developed for studying the immunological reactions of agglutination and protection. By the reaction of agglutination four distinct immunological types and a certain number of unclassifiable strains have been discovered among the 125 strains studied. Individuals of the same type are closely related to one another immunologically, and the different types can be sharply distinguished one from the other. In addition to the four types, study of the reactions of which has been completed, there are in addition two other types, investigation of which is as yet incomplete. The technique of the agglutination reaction demands great care, both as regards the handling of the organisms and the preparation of the medium for their growth. In the medium used by us, a large percentage of strains has grown sufficiently diffusely to permit the preparation of stable suspensions. To what extent continuous growth in this medium has promoted the tendency to diffuseness, and whether the same percentage of freshly isolated strains will grow diffusely, we are as yet unable to say. We have found that by the immunization of sheep a highly specific agglutinating serum is obtained, but that the serum produced from rabbits is not so specific and may show a wider range of crossing, especially in one of the types of streptococcus described. Variations in the specificity of different animal sera have been observed by students of the immunological reactions of meningococcus. In order fully to understand this phenomenon, it would be necessary to compare the specificity of immune sera produced from different species of animals by means of the method of absorption. It is not as yet possible to undertake this kind of an investigation of *Streptococcus hæmolyticus*. The observation has been made, however, that rabbit sera showing non-specific cross-agglutination reactions in general fail to manifest corresponding cross-protection reactions.

Whenever it has been possible to raise the animal virulence of strains of *Streptococcus hæmolyticus*, the evidence obtained from the agglutination tests has been confirmed by that gained from the pro-

tection reaction. In all instances in which this has been done, one reaction has corroborated the findings of the other. The performance of reliable protection tests has been made possible by the production of sufficiently high titer antistreptococcus sera, and by the possibility of raising the animal virulence of a certain number of strains to a high degree. The types of *Streptococcus hæmolyticus* have been noted as Types S 3, S 23, S 60, and S 84, from the serial numbers of the representative strains. This nomenclature is not put forth as a final one, since we realize that probably many other human types exist, to say nothing of the bovine and cheese varieties, and that the proportional distribution of the different varieties pathogenic for man may be very different from that represented by this work. Streptococcus is the largest of all pathogenic groups of bacteria and many years will be required to bring out the information necessary to the perfecting of an adequate classification.

It is of considerable interest that all the members of Type S 60 ferment mannite, and that none of the members of the other groups so far encountered ferments this sugar. A few unclassifiable strains, however, have been found to be mannite fermenters.

This work has cleared up a number of points about *Streptococcus hæmolyticus* which have been in dispute for many years. In the first place, *Streptococcus hæmolyticus* of human origin is not a unit type as was previously supposed, but probably consists of a number of types, at least four of which have been definitely identified. Previous investigators have stated that freshly isolated human strains change their antigenic properties on animal passage, and that the latter procedure for the development of animal virulence gives a common antigenic character to all strains. We have found no evidence to support this contention; in fact immune sera produced with human strains that have never been passed through animals afford a high degree of protection against strains that have received many animal passages. In addition, the antigenic differences between strains of *Streptococcus hæmolyticus* which have been passed through animals are as distinct as those between strains which have not been so passed. The types of *Streptococcus hæmolyticus* studied have been obtained almost exclusively from the respiratory tract and from a limited source of supply, and there is some reason to believe that those which pro-

duce cellulitis, erysipelas, and septicemia may be of somewhat different character. It is, therefore, readily seen that only a beginning has been made in the classification of *Streptococcus hæmolyticus*, and that before the classification is complete and the relative dominance of the different pathogenic varieties determined, much work must be done.

SUMMARY.

1. Immunological differences have been shown to exist between strains of *Streptococcus hæmolyticus* of the human type.

2. Four biological types have been identified by means of the reactions of agglutination and protection.

3. At least two other types have been encountered and the indications are that more exist.

BIBLIOGRAPHY.

1. Schottmüller, H., *Münch. med. Woch.*, 1903, l, 849.
2. Andrews, F. W., and Horder, T. J., *Lancet*, 1906, ii, 708. Holman, W. L., *J. Med. Research*, 1916, xxxiv, 377. Blake, F. G., *ibid.*, 1917, xxxvi, 99.
3. Smith, T., and Brown, J. H., *J. Med. Research*, 1914–15, xxxi, 455. Jones, F. S., *J. Exp. Med.*, 1918, xxviii, 253. Ayers, S. H., *J. Bacteriol.*, 1916, i, 84. Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1919, xxix, 215.
4. Smillie, W. G., *J. Infect. Dis.*, 1917, xx, 45. Cole, R., and MacCallum, W. G., *J. Am. Med. Assn.*, 1918, lxx, 1146. Levy, R. L., and Alexander, H. L., *ibid.*, 1918, lxx, 1827. Opie, E. L., Freeman, A. W., Blake, F. G., Small, J. C., and Rivers, T. M., *ibid.*, 1919, lxxii, 556.
5. Marmorek, A., *Ann. Inst. Pasteur*, 1895, ix, 593. Aronson, H., *Deutsch. med. Woch.*, 1903, xxix, 439. Neufeld, F., *Z. Hyg. u. Infectionskrankh.*, 1903, xliv, 161. Kinsella, R. A., and Swift, H. F., *J. Exp. Med.*, 1918, xxviii, 169. Howell, K., *J. Infect. Dis.*, 1918, xxii, 230.
6. Gay, F. P., *J. Lab. and Clin. Med.*, 1918, iii, 721.
7. Dochez, A. R., and Avery, O. T., *J. Exp. Med.*, 1915, xxi, 114.
8. Lister, F. S., *Publications South African Inst. Med. Research, No. 2*, 1913; *No. 8*, 1916; *No. 10*, 1917.