A STUDY OF MANY STRAINS OF STREPTOCOCCI

Thesis for the Degree of M. S.

Lloyd R. Jones

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A STUDY OF MANY STRAINS OF STREPTOCOCCI WITH SPECIAL REFERENCE TO THE STREPTOCOCCI ISOLATED FROM CASES OF BOVINE MASTITIS.

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T H E S I S

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Submitted to the faculty of the Michigan Agricultural College in partial fulfillment of the requirements for the degree of Master of Science.

By

Lloyd R. Jones.

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THESIS

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INTRODUCTION.

Few, if any pathogenic organisms can lay claim to wider or more multifarious activities than the streptococcus. The list of human and animal diseases with which the streptococci are associated as the main and primary cause is already a long one and probably not yet complete. In addition to their conspicuous role as imitators of very diverse pathological conditions, streptococci are very often present in mixed or secondary infections. Streptococci occur in the healthy human subject both on the skin and in those cavities which open on the surface of the body as the alimentary canal and nose. They are to be found in the saliva and feces and have a wide distribution in air, soil and well water in nearly all pathological conditions of the throat of whatever nature streptococci are to be found either as the primary cause or as an associated infective agent. These organisms are nearly constantly present in secondary infections in scarlet fever.

The epidemic of streptococcus sore-throat that prevailed in Chicago during the winter of 1911-12 was definitely traced to one dairy. Among the herd supplying this dairy was found a number of cases of mastitis. Rosenow (1) reports that a type of streptococcus slightly hemolytic and corresponding to the pyogenes type, only after artificial cultivation, occurred in predominating numbers as the etiologic factor of the sore-throat epidemic. In Boston (2) and in Baltimore (3) likewise,

epidemics have occurred corresponding to the one in Chicago, milk borne and with similar streptococci.

Davis and Capps (4) of Chicago in experimental work have caused mastitis in the bovine by the injection of hemolytic streptococci of human origin. These with a number of similar observations tend to show relationship in the etiology of septic sore-throat and bovine mastitis. Observations would indicate that both conditions are caused by the same organism and it is the purpose of this paper to present a study of the streptococci collected from both sources and other available strains.

Review of Literature.

Streptococci were seen in unstained pus by Klebs in 1872. Several years later Koch (5) demonstrated them in stained sections and in inflammatory exudates. Pasteur (6) appears to have been the first to cultivate the streptococci from cases of puerperal fever and to differentiate them from staphlyocci both morphologically and by the character of the lesions which they excite. Rosenbach (7) studied the organism in great detail and introduced the name Streptococcus pyogenes.

The question of the differentiation of the streptococci which soon arose after the first description of the organisms is still unsettled. Numerous researches for the past twenty years having failed to present any method or system for proving or disproving the identity of the many strains of streptococci. The large variety of pathological conditions to which the organism can give origin and its occurence as a saprophyte

on the healthy human tissues has for a long time attracted the attention of bacteriologists. "One and the same strain of streptococcus may at different stages in its career produce now a localized suppuration and now no effects at all," say Andrewes and Horder.

Besredka says that, "it may therefore be accepted as an established fact that a streptococcal infection may assume different clinical manifestations depending upon the resistance of the person infected and the source whence the organism was originally derived."

Considerable discussion has arisen concerning the unity or plurality of types included within the species known as Streptococcus pyogenes. Marmorek (8) and others have stoutly maintained the "Einheit" theory. Considerable evidence in favor of this view has been advanced by Koch and Petruschky (9) who showed that a streptococcus obtained from a fatal puerperal sepsis caused erysipelas in a rabbit when it was injected subcutaneously, ~~peritonitis when it was injected subcutaneously~~, peritonitis when injected intra-peritoneally and septicaemia when injected intravenously. The conclusions were that the type of lesions produced by Streptococcus pyogenes depended largely upon the virulence of the culture, the tissue invaded and the number of organisms. Additional evidence of the "Einheit" of streptococci has been brought forward by Rosenow (10) who states that he has changed streptococci to pneumococci and back again by special methods of culture and animal inoculation.

The important question for the moment is, Do these changes of virulence, et cetera, exhibited by the streptococcus

influence the diagnostic aspect of the question? Theobald Smith has admirably summed up the present status of the subject in the following words: "Spontaneous changes in the cultural characteristics of the streptococcus do not proceed rapidly enough, if they go on at all, to interfere with current bacteriological methods. Tendencies toward slow changes may be used as further valuable distinguishing characters" (11).

The marked pleomorphism of the streptococcus also led many observers to classify these organisms according to their microscopic appearance. Von Lingelsheim has made the morphological distinctions paramount and thus proposed the groups Str. longus and Str. brevis.

The English bacteriologists were the pioneers in investigating the fermentation activities of the streptococci with a view to ascertaining whether essential differences such as would be of value in classifying could be observed. Working upon the assumption that the fermentative powers are biological characters of fundamental importance, Gordon and Andrewes and Horder employed certain fermentable substances in culture media. A complete discussion of the grouping according to action upon carbohydrates and allied substances prefaces the "Fermentation reactions" given under experimental work.

Method of Investigation.

The cultures from milk samples have been isolated from plates by picking off colonies of characteristic appearance, subculturing and subsequently replating, after microscopic examination in order to determine purity of the culture.

Throat swabs from sore-throat and scarlet fever patients were immersed in five c.c. of sterile physiological salt solution. The salt solution blank with inoculum was shaken and streaks from the suspension made on the surface of human blood-agar plates. After twenty four hours incubation at 37°C. the characteristic colonies were picked off and sub-cultured. The salt solution blank dilutes the inoculum to such an extent that ordinarily colonies are distinct upon the plate, convenient for removing. The use of the human blood-agar plate offers two advantages, first, because streptococci grow vigorously upon such a medium and second, it affords an immediate comparison of relative numbers of hemolyzing and non-hemolyzing types present in the infection.

In the case of pus samples, loops of the material have been placed in salt solution blanks and the method followed as outlined for throat swabs.

Thirty-eight strains have been studied culturally and twenty two strains employed in the immunological reactions. The source from which the cultures have been derived is given herewith:

1.	Diseased udder.	Chronic mastitis.
2.	do	do
3.	do	do
4.	do	Acute mastitis.
5.	do	Cow had had an attack of mastitis some time previous. Milk apparently normal.
6.	do	Chronic mastitis.
7.	do	Samples from Grand Rapids, Mich.

8. Diseased udder. Sample from Grand Rapids, Mich.

9. do Acute mastitis.

10. do do

11. do Chronic mastitis.

12. do do

13. do These four strains were isolated from samples of "gargety" milk
14. do from Portland, Mich. Mastitis of an infectious nature assumed
15. do the proportion of an outbreak among the herd. A few of the
16. do cases were fatal.

17. Streptococcus lacticus. Laboratory stock culture.

18. Streptococcus. Sore throat.

19. do do

20. do Sore throat with La Grippe.

21. do Sore throat with Scarlet fever.

22. do Sore throat.

23. do do

24. do do

25. do From same patient as No. 24.

26. do Sore throat of chronic type.

27. do Normal throat, patient had an abscess of the gum.

28. do Normal throat.

29. do hemolyticus. Stock culture.

30. do pyogenes. Stock culture.

31. Streptococcus from sputum.

32. Streptococcus stock culture.

33. Streptococcus pyogenes. Pyemic arthritis of bovine.

34. Streptococcus from endocarditis. Human.

35. Streptococcus hemolyticus, stock culture.

36. Streptococcus metachromatos, stock culture.

37. Streptococcus isolated from contaminated milk.

38. Streptococcus isolated from equine peritonitis. Sequel of castration operation, organism recovered in pure culture.

During the first part of this study the cultures were kept growing in dextrose broth containing one per cent of calcium carbonate which served to prevent the accumulation of acid. Later bovine blood-agar was used. The method of preparation of this medium was as suggested by Bernstein (12). One hundred cubic centimeters of blood were drawn from the jugular vein of a cow into a 200 c.c. Erlenmeyer flask containing 7.5 c.c. of a one per cent solution of ammonium oxalate and 125 c.c. of formalin (forty volume strength). The blood mixture was shaken for two minutes. The ammonium oxalate prevents clotting, and thirty minutes are required for sterilization. The blood was then distributed into smaller flasks and twice its volume of sterile 0.9 per cent salt solution added. This gives a dilution of 1 to 2400 of the formalin. The small flasks were sealed and kept in the ice box until needed. Plain agar (0.6 per cent acid) tubes were thoroughly melted and then cooled to between 50° and 60°C. and one part of the diluted blood was added to fifteen parts of the media, which gave an approximate dilution of 1 to 36000 of the formalin which is not strong enough to interfere with the growth of organisms upon the medium.

After slanting, the tubes were incubated to insure sterility. Transfers of the stock cultures were made at intervals averaging seven days.

In undertaking the immunological reactions, twenty two strains of quite definite fermentation characteristics were

selected for rabbit inoculation. Pre-injection tests were made to obtain a record of non-specific reaction both for agglutination and fixation of complement. Naturally, of primary importance was the perfection of a technic which would give uniform results. After the period of immunization blood samples were taken which were tested against the homologous serum and also against the twenty-one heterologous sera. This work was thought worthy of undertaking from a standpoint of perfecting a suitable technic and its possible application in differentiation of strains or in determining the specificity of immune sera to their respective antigens.

EXPERIMENTAL WORK.

Morphological and Cultural Characteristics.

The streptococcus group comprises those spherical bacteria in which as multiplication proceeds the successive planes of division are parallel and the individual cells remain adherent in longer or shorter chains. The term, streptococcus, therefore is purely morphological. No help in the classification of the streptococci is afforded by the form of the individual cell, since under favorable conditions all appear as regular spheres. Irregular oval forms occur at times, particularly in cultures freshly isolated from the throat but the form usually becomes normal after cultivation. The streptococci exhibit a range in size from 0.5 to 1 micron in diameter with considerable variation between individual cells in the same culture. No relation has been found to exist between size and age of the culture or conditions of cultivation.

The organisms remain adherent in chains which vary in length from four to a hundred or more elements, in which a

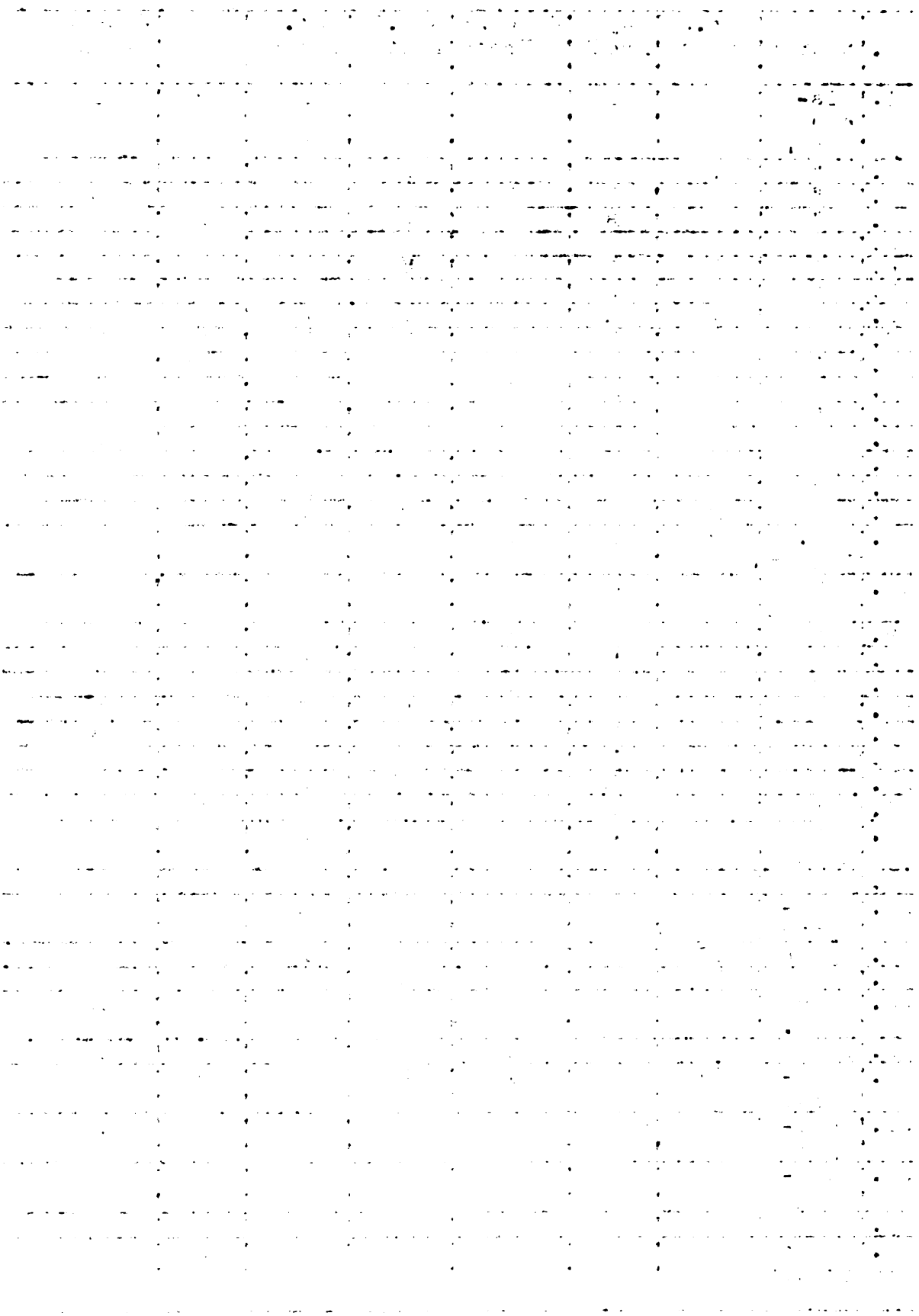
definite association of the cocci in pairs with their proximate sides flattened is occasionally observed. The number of elements in the chain varies somewhat according to the origin of the culture. It has been observed that streptococci freshly isolated from lesions tend to occur in longer chains, while those organisms which grow habitually upon the normal surfaces and muceus membranes of the body appear more frequently in shorter chains.

Hopkins and Lang (13) in an extended study of many strains based their records of chain lengths on smears of the sediment of twenty-four hour dextrose broth cultures. Those forming chains of less than ten members were classed as brevis, those of ten to fifteen members as Medius and those of more than fifteen were classed as longus. Those strains forming very long chains covering several fields have been designated as longior. A similar plan has been used in the chain length observations in this study. In most smears both long and short chains have been found so it is difficult to utilize this classification but these terms are of value mainly in a comparative way.

Table I gives the chain lengths for the strains used in this study. Observations were made from smears of the sediment of a twenty-four hour dextrose broth culture and from the water of condensation of a four day blood-agar culture.

Table I.

No.	Source	Mar. 30 Broth	May 3 Broth	June 28 Broth	Aug. 15 Broth	Mar. 30 Broth	May 3 Broth	June 20 Broth
1.	Diseased udder	L	L	L	M	M	B	B
2.	"	L	L	L	L	M	M	B
3.	"	L	L	L	M	B	B	B
4.	"	B	B	B	B	B	B	B
5.	"	B	B	B	B	B	B	B
6.	"	L	L	L	M	L	M	B
7.	"	B	B	B	M	B	B	B
8.	"	M	M	M	M	M	M	B
9.	"	LL	LL	LL	L	M	M	M
10.	"	L	L	L	M	L	M	B
11.	"	M	M	L	B	M	B	B
12.	"	M	M	L	M	B	B	B
13.	"	M	M	M	M	M	B	B
14.	"	L	L	M	B	M	B	B
15.	"	B	M	M	B	M	B	B
16.	"	M	B	M	B	M	B	B
17.	Str. Lacticus	B	B	B	B	B	B	B
18.	Sore throat	M	M	L	L	M	M	B
19.	"	B	B	B	B	B	B	B
20.	"	M	M	M	M	M	M	B
21.	"	M	M	L	L	B	B	M
22.	"	M	M	B	B	B	B	B
23.	"	B	B	B	B	B	B	B
24.	"	LL	LL	LL	LL	L	M	M
25.	"	L	LL	LL	LL	B	M	B
26.	"	B	B	B	M	B	B	B
27.	Normal throat	B	M	B	B	B	B	B
28.	"	B	M	B	M	M	B	B
29.	Hemolyticus	M	M	L	M	M	B	B
30.	Pyogenes	LL	L	L	L	B	B	B
31.	Sputum	M	B	M	M	B	B	B
32.	Stock Cult.			L	B		B	B
33.	Pyogenes	M	M	B	B	B	B	B
34.	Endocarditis	LL	LL	L	LL	M	M	B
35.	Hemolyticus			M	M			M
36.	Metachromatos			M				B
37.	Milk	B	B	B	B	B	B	B
38.	Equine peritonitis	M	M	M	M	B	B	B



It appears from the foregoing table that the record of chain length does not afford a reliable basis for classification primarily because cultural conditions greatly modify the length of chains formed.

Previously, a long chain streptococcus has been considered as more virulent than one of short chain type. In this study it is to be noted that saprophytic strains are essentially of short chain type, but not all strains from active lesions are of the long chain type. Numbers 33 and 38, isolated from active lesions and of active hemolytic properties show short chain type in contradistinction to the generally accepted theory regarding the relationship of long chain formation and virulence.

There is in general a tendency for shorter chain formation after a prolonged period of artificial cultivation which tends to be more marked upon solid media than with broth cultures, however there are frequent exceptions to that tendency for in a few instances the reverse difference has been noted.

Typical streptococci are non-motile, non-flagellated and do not produce true endospores. Ellis (14) reports the finding of spores and flagella in streptococci. The experiments carried on by Ellis would rather indicate the presence of specially resistant cells in old cultures of the cocci. His figures are by no means conclusive as to the existence of true spores and, in the absence of any observation of germination of streptococcus spores, they can scarcely be said to exist. Streptococci may exhibit from time to time cells which are decidedly larger than their fellows.

They have been designated by Heuppe as arthrospores. These arthrospores appear to have no unusual resisting powers, and they are in no sense to be regarded as true spores. It is very probable they are involution forms. C.E.A. and A.E. Winslow (14) found that one of the fundamental differences between the parasitic and the saprophytic cocci was their behavior to the Gram stain. The parasitic form usually decolorized. These observations were corroborated by Kligler (15) who in reviewing the coccacae in the collection at the Museum of Natural History found that of the saprophytic types seventy four per cent were Gram negative and none per cent were Gram positive and the remaining seventeen per cent were variable. The cultures used in this study were found to be Gram positive when smears were made from four day agar cultures. No deviation from this rule was observed.

Andrewes and Horder (17) consider the characteristic growth in litmus milk one of the most significant tests and their differentiation between the Streptococcus pyogenes group and the anginosus group is based partly on this criterion. Their six groups of the streptococci are reported as giving the following changes in litmus milk:

Equinus	- fails to clot milk.
Mitis	- acidifies milk without clotting.
Pyogenes	- acidifies milk without clotting.
Salivarius	- clots milk.
Anginosus	- clots milk.
Fecalis	- clots milk.

The only changes which the cocci effect in milk are the production of acid or alkali, coagulation and decolorization

of the litmus. When the organism is most active it uses up the oxygen and reduces the litmus which is accordingly decolorized and conversely when activity grows less, oxygen diffuses from the surface making the litmus pink again. Coagulation is dependent upon the amount of acid formed. No casein-digesting enzymes are found among the streptococci and no gas or odor is produced.

In isolating the strains of streptococci from the udder, samples have been taken from udders showing certain evidence of infection. Observations were made as to the relative prevalence of streptococci as compared with other organisms and of relative numbers of hemolytic and non-hemolytic types of the streptococcus. Naturally considerable apprehension existed in regard to the possibility of strains so isolated, corresponding in type to the streptococcus lacticus (Kruse) a saprophytic type in many cases responsible for the souring of milk. Sherman, Evans and Hastings (16) have suggested that differentiation is roughly made by characteristic growth on litmus milk. They state that the long chain type of streptococci usually curdle the milk and may reduce the litmus after the curdling, but the color is never completely reduced. With Streptococcus lacticus cultures, however, the reduction of the litmus preceeds the curdling and is complete beneath the sharply defined surface layer. Streptococcus lacticus also lacks the tendency to form long chains.

With the fermentation reactions it will be noted that Streptococcus lacticus cultures correspond very closely to the characteristics manifested by the Hyogenes group, with the

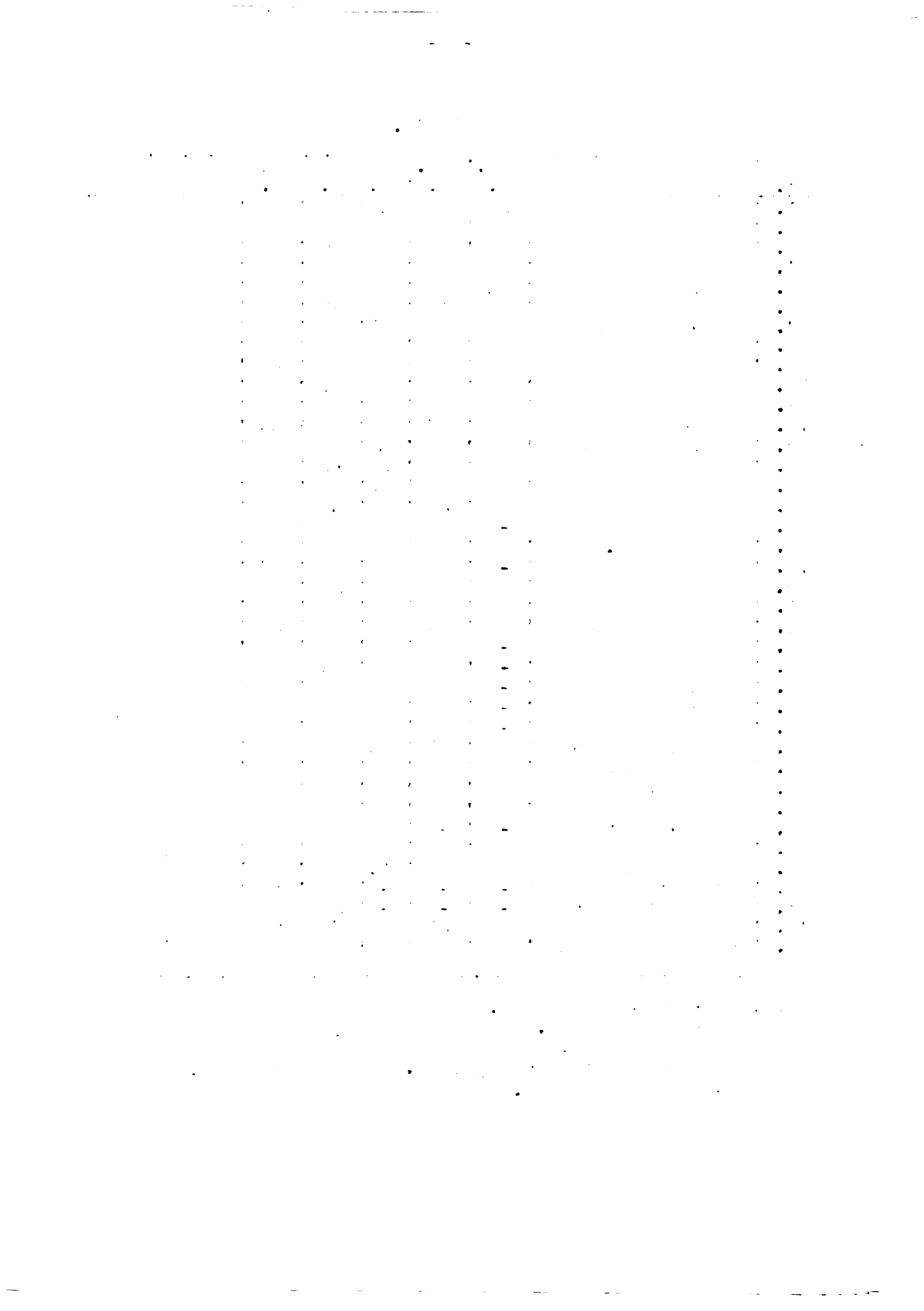
exception that the lacticus cultures are never hemolytic while cultures of the pyogenes type are invariably ~~so~~ so, a feature paramount in differentiation.

In Table II are given the results of four days incubation in litmus milk. In no instance were changes noted after the fourth day which were to be regarded as of bacterial origin.

Table II.

No.	Source	Mar. 1.	Mar. 28.	May 3.	June 6.	July 7.
1.	Diseased udder	AC	AC	AC	AC	AC
2.	do do	AC	AC	AC	AC	AC
3.	do do	AC	AC	AC	AC	AC
4.	do do	AC	AC	AC	AC	AC
5.	do do	SL	SL	SL	SL	SL
6.	do do	AC	AC	AC	AC	AC
7.	do do	SL	SL	SL	SL	SL
8.	do do	A	A	A	A	AC
9.	do do	AC	AC	AC	AC	AC
10.	do do	AC	AC	AC	AC	AC
11.	do do	AC	AC	AC	AC	AC
12.	do do	AC	AC	AC	AC	AC
13.	do do	AC	AC	ACC	AC	AC
14.	do do	AC	AC	AC	AC	AC
15.	do do	AC	AC	AC	AC	AC
16.	do do	AC	AC	AC	AC	AC
17.	do do	-	A	SL	SL	SL
18.	Sore throat	O	C	AC	AC	AC
19.	do do	-	AC	AC	AC	AC
20.	do do	O	A	A	A	AC
21.	do do	A	A	A	A	A
22.	do do	AC	AC	AC	AC	AC
23.	do do	-	A	A	A	A
24.	do do	-	C	C	AC	AC
25.	do do	-	C	C	AC	AC
26.	do do	-	O	O	O	O
27.	do do	-	A	O	O	O
28.	Normal throat	AC	AC	AC	AC	AC
29.	Hemolyticus	A	A	A	AC	AC
30.	Pyogenes	A	A	A	A	AC
31.	Sputum	A	A	A	A	A
32.	Stock culture	-	-	A	A	A
33.	Pyogenes	O	AC	A	A	A
34.	Endocarditis	AC	AC	AC	AC	AC
35.	Hemolyticus	-	-	-	O	A
36.	Metachromatos	-	-	-	AC	AC
37.	Milk	SL	SL	SL	SL	SL
38.	Equine Peritenitis	A	A	A	A	AC

Note: A = Acid production.
C = Coagulation.
O = No change.
SL = Streptococcus lacticus. type of reaction.
- = Test not made.



During the period of cultivation as indicated in Table II, twenty six strains continually gave the same reaction when plated in litmus milk. The remaining twelve acquired new characteristics which, for most of them were the formation of acid and coagulum. A majority of the udder strains gave regularly an acid coagulum type of reaction.

Among the sore-throat cultures which, according to Andrewes and Horder's classification, should clot milk without producing acid there were but two that so reacted. From a standpoint of classification the character of the growth in litmus milk can scarcely be considered as satisfactory.

According to Andrewes and Horder's classification the growth of streptococci on gelatin at 20°C. varies among the different groups as follows:

equnius -feeble.
mitis -well.
pyogenes -well.
salivarius -variable.
anginosus -fails.
fecalis -well.

Gelatin cultures of the streptococci invariably present the filiform appearance with very closely aggregated colonies. No liquefaction was ever observed. After several generations on artificial media there tends to be a more abundant growth on gelatin. Table III, gives results of stab inoculations into nutrient gelatin after incubation for four days at room temperature, approximately 20°C.

Table III.

No.	Source.	Mar. 20	Apr. 20	May 5	June 20	August 15
1.	Diseased udder	+	+	+	+	+
2.	do	+	+	+	+	+
3.	do	+	+	+	+	+
4.	do	-	+	+	+	+
5.	do	+	+	+	+	+
6.	do	+	+	+	+	+
7.	do	+	+	+	+	+
8.	do	-	-	-	-	-
9.	do	+	+	+	+	+
10.	do	+	+	+	+	+
11.	do	+	+	+	+	+
12.	do	+	+	+	+	+
13.	do	+	+	+	+	+
14.	do	+	+	+	+	+
15.	do	+	+	+	+	+
16.	do	+	+	+	+	+
17.	Str. lacticus			+	+	+
18.	Sore throat	-	+	+	+	+
19.	do	-	-	-	-	-
20.	do	-	-	-	+	+
21.	do	-	-	+	+	+
22.	do	-	-	-	+	+
23.	do	-	-	Sl	Sl	Sl
24.	do	-	Sl	Sl	+	+
25.	do	-	-	Sl	+	+
26.	do	-	-	-	+	+
27.	Normal throat	+	+	+	+	+
28.	do	-	-	-	+	+
29.	Hemolyticus	-	-	+	+	+
30.	Pyogenes	+	+	+	+	+
31.	Stock culture	0	0	0	+	+
32.	Sputum	-	-	+	+	+
33.	Pyogenes	+	+	+	+	+
34.	Endocarditis	+	+	+	+	+
35.	Hemolyticus	0	0	0	+	+
36.	Metachromatos	0	0	0	Sl	+
37.	Milk	+	+	+	+	+
38.	Equine peritonitis	+	+	+	+	+

Note: + = growth.
- = no growth.
Sl = slight growth.

All but two of the seventeen udder strains continued to grow well on gelatin, which would permit of their classifying with the pyogenes type according to Andrewes and Horder. Of the sore throat strains there was but one that

grew in gelatin immediately after isolation. As a supplementary test, particularly with reference to differentiation between the pyogenic and anginosus groups, characteristics of growth upon gelatin appear to be of very considerable importance.

Hemolysis.

Hemolytic determinations on blood agar plates introduced by Schottmüller (39) is perhaps the most widely used means of differentiating the streptococci. Originally, the method of determination consisted in inoculating the hearts blood of a rabbit dying of streptococcal septicaemia directly into whole serum and observing the height to which the ring of dissolved hemoglobin arose.

According to Davis (18) not enough emphasis has been placed on the property of hemolysis in the general study of streptococci. Particularly, with strains isolated from diseased udders as the hemolytic property is an invaluable means of distinguishing between saprophytic types and many of the pathogens.

Numerous workers have attempted the classification of the streptococci solely upon their characteristic reactions upon some sort of blood media. "Hemolytic," "viridans" and "non-hemolytic" are terms that are generally accepted as characteristic of certain streptococci. All observers are agreed upon the existence of the hemolytic and non-hemolytic types, the former producing that clear transparent zone peripheral to the colony; but for the second or viridans group there are many observations that tend to invalidate this classification. "The hemolytic power

possessed by the streptococci is another of the subtle properties developed in direct response to the bio-chemical conditions of the host," says Winslow.

It is to be understood that the hemolytic streptococci do not constitute a single variety of genus. Hemolysis is a property common to a number of kinds of streptococci that might differ from one another in a number of other properties. It is variable at least within certain limits, but nevertheless sufficiently stable to be a very useful property for many practical purposes.

Some workers believe the greenish coloration of the viridans colony to be due to the action of the cocci upon the muscle sugar present since in sugar-free media the action may not appear and on glucose blood agar even the hemolytic types may show this greenish coloration. Nevertheless, it is an observed fact that greenish colonies do result upon the cultivation of certain strains of organisms, and at the present time this type of organism is recognized as Streptococcus viridans though we have not at present any binding or standard classification.

Methemoglobin is an intermediate product in the reduction process of oxyhemoglobin and it is this reduction that takes place in the formation of the greenish coloration. This phenomenon occurs only when the streptococci are living and only in the presence of certain nutritive materials and is to be explained by the alteration of the oxidation processes by the organisms in the vicinity of the red blood cells and not to the production of any injurious substances capable of isolation. Further, we can also see at once that

the pathologic effects of bacteria on other tissue cells may be due to disturbance in the oxidation in the immediate neighborhood of the cells and not necessarily to the action of any excreted or secreted poison. It is believed that this theory may be applicable to the effects caused by the Streptococcus viridans because the lesions produced by it are prone to be localized and associated with the presence of the organisms in the lesion. The evidence is also supported by an elimination of other factors: As, methemoglobin is produced in the presence of sugars that are not fermented and so it is not dependent upon any intermediate or and products of sugar fermentations; it is not due to the production of carbon dioxide as this gas produces in hemoglobin a cherry red pigment; it is not due to the production of acid as this reaction is somewhat accelerated when acids are removed by potassium phosphate. The phenomenon of the alteration of the oxidation and reduction processes when stimulated still further results in an active hemolysis of which the hemolytic group is capable according to one explanation. And again, the hemolytic area or clear zone surrounding a colony may be due to certain proteolytic enzymes which emenate from the colony and attack the red blood cell, either changing the state of the membrane or severing the relations of the hemoglobin from the stroma of the erthrocyte.

In determining hemolysis in the laboratory, the blood of various species has been used, though only a few comparative studies have been made. Guinea pig blood has

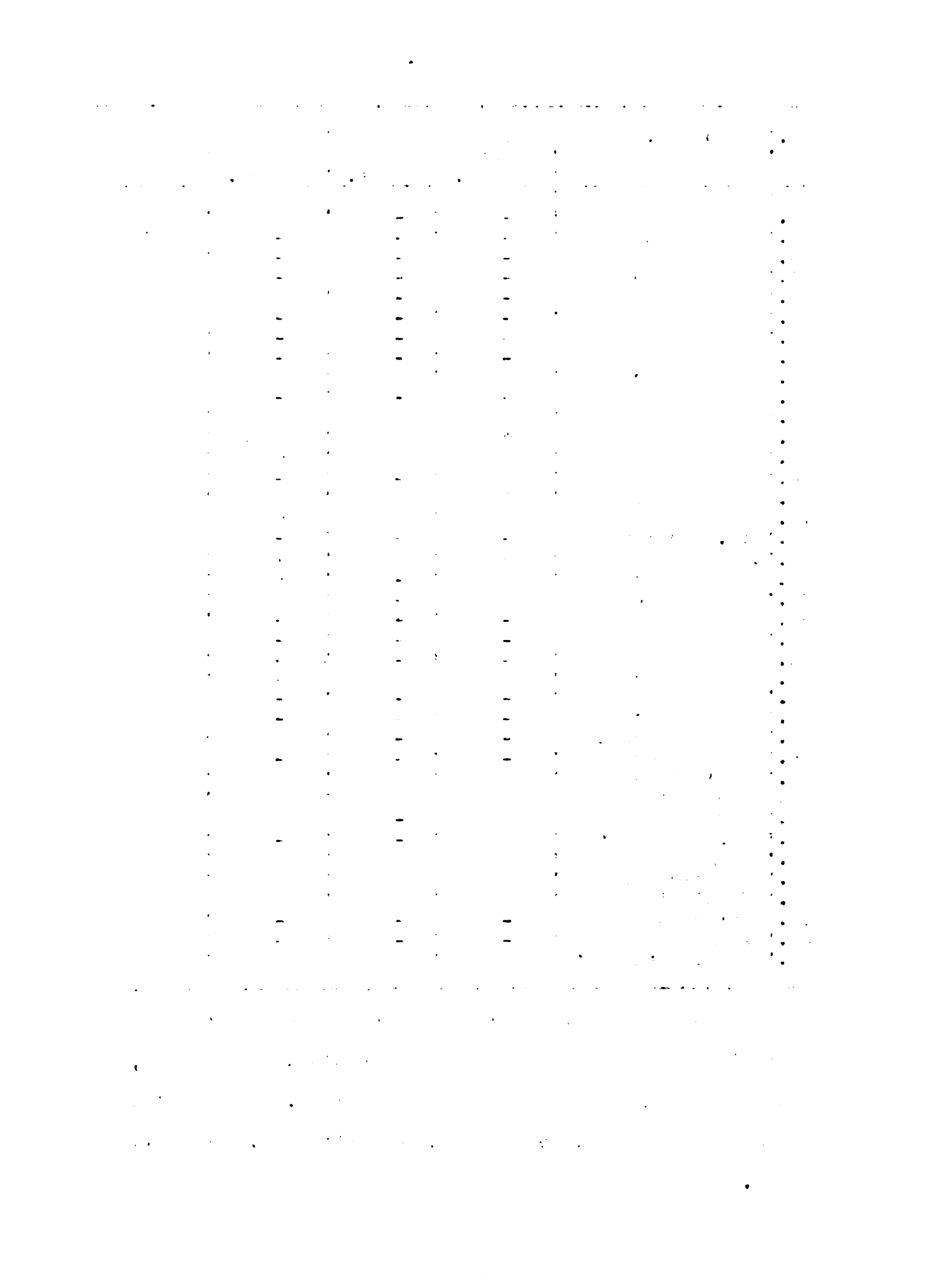
been reported by a number of observers as unsuitable because of the rapid disintegration of the corpuscle. Kerner (19) found that the corpuscles of the dog are most easily hemolyzed while human and frog are the most resistant. Becker (20) in a comparative study of sheep, goat, horse, rabbit and human bloods found that variations were especially marked in the macroscopic appearance of the hemolytic zone. Irregular differences were noticed in the extent of the hemolysis, with the various kinds of blood. Methemoglobin was formed most quickly on rabbit blood. Human blood appeared to resist hemolysis by weakly hemolytic strains better than any of the others.

In the routine preparation of nutrient blood agar for maintaining the stock cultures, defibrinated bovine blood with one per cent ammonium oxalate solution and sterilized with one tenth per cent of formalin as suggested by Bernstein and Epstein (12) was used. A series of plates was poured with this bovine blood agar, so preserved, and was inoculated from the same culture as a series of the human blood agar plates. A comparative record of the results is given in Table IV.

Table IV.

No.	Source.	Human blood 24 hrs.	Bovine blood 24 hrs.	Bovine blood 24 hrs.
1.	Diseased Udder	-	-	+
2.	do	-	-	-
3.	do	-	-	-
4.	do	-	-	-
5.	do	-	-	-
6.	do	-	-	-
7.	do	-	-	-
8.	do	-	-	-
9.	do	+	-	+
10.	do	-	-	-
11.	do	+	+	+
12.	do	+	+	+
13.	do	+	+	+
14.	do	+	-	-
15.	do	+	+	+
16.	do	+	+	+
17.	Str. Lacticus	-	-	-
18.	Sore throat	+	+	+
19.	do	+	-	+
20.	do	+	-	+
21.	do	-	-	-
22.	do	-	-	-
23.	do	-	-	-
24.	do	V	-	-
25.	do	-	-	-
26.	do	-	-	-
27.	Normal throat	-	-	-
28.	do	-	-	-
29.	Hemolyticus	+	+	+
30.	Pyogenes	+	+	+
31.	Sputum	+	-	+
32.	Stock culture	+	-	-
33.	Pyogenes	+	+	+
34.	Endocarditis	+	+	+
35.	Hemolyticus	+	+	+
36.	Metachromatos	-	-	-
37.	Milk	-	-	-
38.	Equine peritonitis	+	+	+

In some instances hemolysis was noted on the bovine blood agar plates corresponding to the human blood agar plates, but only after forty eight hours incubation. The results do not agree to a sufficient extent to merit the use of bovine blood.



Lyall (21) points out that the determination of hemolysin production on blood agar plates is of necessity in no degree quantitative since the hemolysis is subject to so many variations, that arise from varying concentrations of the blood present, hemolysis due to acid production and inhibition of hemolysis resulting from the presence of certain carbohydrates. To obviate these difficulties and make possible a quantitative determination Lyall added a definite amount of broth culture to one c.c. of a five per cent suspension of washed sheep cells and incubated in a water bath for one hour at 37°C. This method, he considers, when applied to streptococci gives three types of reaction. First, those giving complete solution of the erythrocytes. Second, characterized by a change in color of the suspension from a bright red to a dark brown, due to a transformation of oxyhemoglobin into methemoglobin. In the third type there is no solution of cells or change in color.

The cultures reported in this study were tested for hemolysis according to the method of Lyall. One half cubic centimeter of an eighteen hour calcium carbonate-dextrose-broth culture was added in each instance to one cubic centimeter of a five per cent suspension of washed sheep cells and placed in 37°C. water-bath for one hour. Thinking that the reaction might be more easily effected with a more dilute suspension of the cells, a series of tubes containing a two and one-half per cent suspension of cells was run simultaneously. Human blood agar plates were inoculated from the same cultures and incubated at

37°C. for twenty four hours in order to have an exact comparison between the two methods. The results of the three series are given in Table V. Methemoglobin production appears to be the predominant type of reaction and without analogy to the type of reaction on human blood medium. The results from the Lyall method do not correspond to the known type of the organism.

Table V.

No.	Source.	Lyall Method. 5 per cent Susp. cells.	Lyall Method. 2.5 per cnet Susp. cells.	Human-blood agar plate.
1.	Diseased udder	-	-	-
2.	do do	-	-	-
3.	do do	-	-	-
4.	do do	-	-	-
5.	do do	M	-	-
6.	do do	-	-	-
7.	do do	M	M	-
8.	do do	-	-	-
9.	do do	M	M	+
10.	do do	M	M	-
11.	do do	M	-	+
12.	do do	M	-	S1
13.	do do	M	-	+
14.	do do	-	-	+
15.	do do	M	-	+
16.	do do	M	-	+
17.	Str. Lacticus	M	-	-
18.	Sore throat	+	+	+
19.	do do	M	-	-
20.	do do	-	-	-
21.	do do	-	-	-
22.	do do	M	M	-
23.	do do	M	M	-
24.	do do	M	-	S1
25.	do do	M	-	+
26.	do do	M	-	+
27.	Normal throat	+	-	+
28.	do do	M	M	+
29.	Hemolyticus	+	+	+
30.	Pyogenes	+	+	+
31.	Sputum	+	+	+
32.	Stock culture	+	-	+
33.	Pyogenes			+
34.	Endocarditis	+	+	+
35.	Hemolyticus	-	-	-
36.	Metachromatos	M	-	-
37.	Milk	M	-	-
38.	Equine peritonitis	-	+	+

In order to obtain a comparison of the ability of the various strains to hemolyze red blood cells and of the possibility of green pigment production (common to Schottmüller's group of Streptococcus viridans)upon blood media the following technic was used as recently suggested by Becker (20) and which from the experimental evidence at hand is the most satisfactory indicator of the ability of organisms to effect the dissolution of erythrocytes.

Standard agar, as used in water analysis, containing 1.5 per cent agar, one per cent peptone and made one per cent acid to phenol-phthalein was used. The agar after melting was cooled to between 50 and 60 C. and one cubic centimeter of sterile human blood added for each six cubic centimeters of the agar base, and the whole thoroughly mixed by gentle rotation. Approximately seven cubic centimeters were used for each plate. One drop of a twenty four hour broth culture was placed in five cubic centimeters of sterile salt solution which served as a diluting agent for the inoculum giving isolated colonies upon the plate. Surface streaks were made and results noted after twenty four hours incubation at 37°C. Isolated colonies only were used as a basis in detecting hemolysis.

Most workers advise the use of defibrinated human blood in preparing the plates. The writer has observed that the possibility of contamination can be minimized by permitting the blood to flow from the median cephalic vein of the arm directly into the liquefied agar which has been cooled. As the tube is immediately poured sufficient time does not elapse for coagulation to take place. The hemolytic reactions of the cultures in this study upon human blood agar are given in Table VI.

Table VI.

No.	Source	April 5	May 3	June 6	July 9	August 15
1.	Diseased udder	V	-	-	-	-
2.	do do	-	-	-	-	-
3.	do do	+	-	-	-	-
4.	do do	-	-	-	-	-
5.	do do	-	-	-	-	-
6.	do do	-	-	-	-	-
7.	do do	-	-	-	-	-
8.	do do	-	-	-	-	-
9.	do do	+	+	+	+	Sl
10.	do do	-	-	-	-	-
11.	do do	+	+	+	+	+
12.	do do	+	+	+	Sl	Sl
13.	do do	+	+	+	+	+
14.	do do	+	+	+	+	-
15.	do do	+	+	+	+	+
16.	do do	+	+	+	+	-
17.	Str. Lacticus	O	O	-	-	-
18.	Sore throat	+	+	+	+	+
19.	do do	V	V	-	-	-
20.	do do	+	+	+	-	-
21.	do do	+	Sl	-	-	-
22.	do do	O	O	-	-	-
23.	do do	-	-	-	-	-
24.	do do	+	+	Sl	Sl	Sl
25.	do do	-	-	-	-	-
26.	do do	-	-	-	-	-
27.	Normal throat	+	-	-	-	-
28.	do do	-	-	-	-	-
29.	Hemolyticus	+	+	+	+	+
30.	Pyogenes	+	+	+	+	+
31.	Sputum	+	+	+	+	Sl
32.	Stock culture	O	O	+	+	Sl
33.	Pyogenew	+	+	+	+	-
34.	Endocarditis	+	+	+	+	+
35.	Hemolyticus	O	O	+	+	+
36.	Metachromatos	O	-	-	-	-
37.	Milk	-	-	-	-	-
38.	Equine peritonitis	+	+	+	+	+

In Table VI it will be noted that out of twenty one cultures which on April fifth were recorded as giving hemolysis on human-blood agar only nine gave the same reaction on August fifteenth, after a period of slightly more than four months artificial cultivation. Apparently the property of hemolysis is not to be considered as a

permanent characteristic of some streptococci. Immediately the question arises: Can streptococci acquire the property of hemolysis when subjected to very favorable conditions as animal passage? During this period of study none have acquired the property. Further investigation is necessary to elucidate this point.

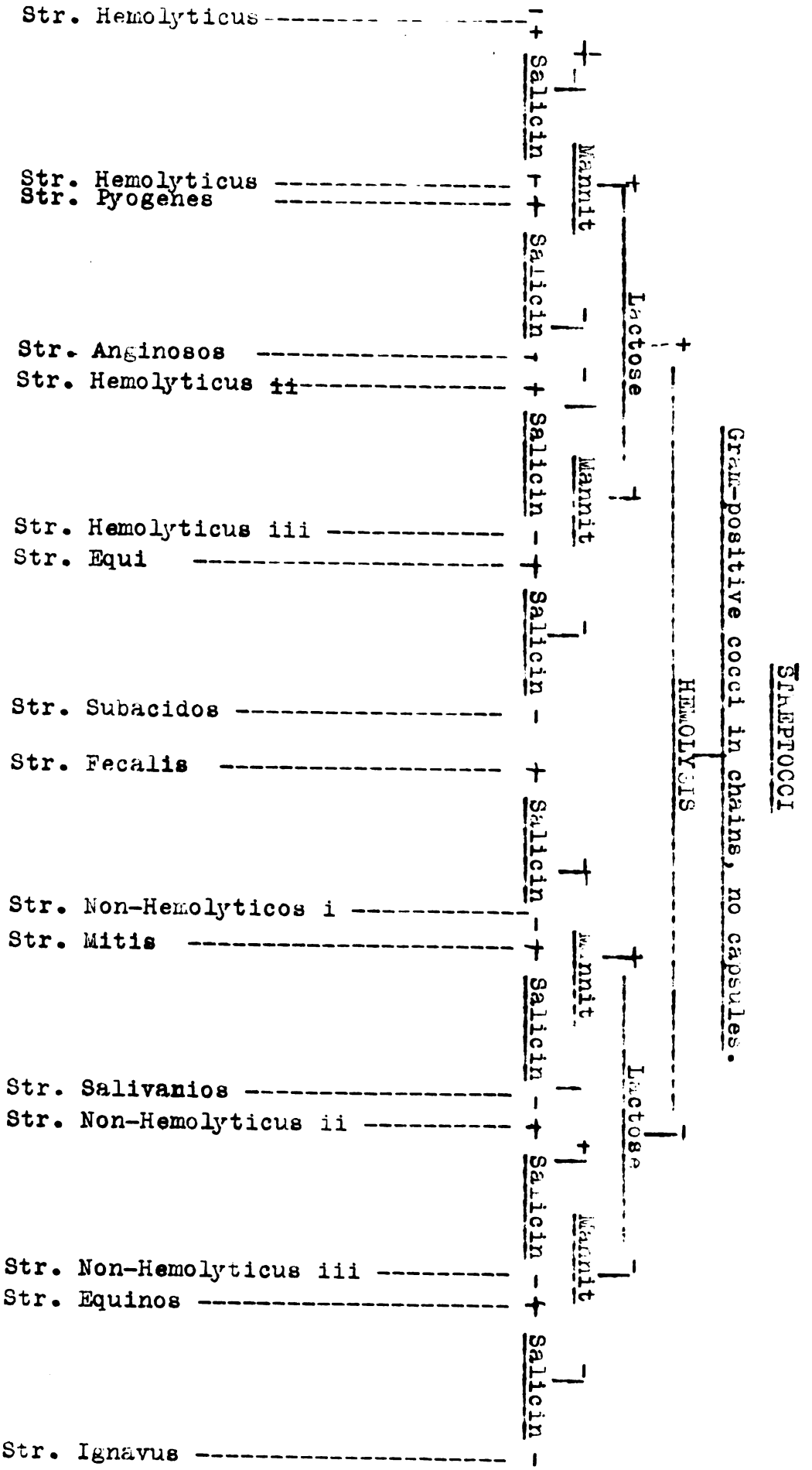
Fermentation of Saccharine Broths.

The application of the carbohydrate-splitting powers to classification, which proved of such value in the typhoid, coli, and dysentery group of bacilli, has been attempted in the case of the streptococci in a desultory way by many observers but very thoroughly by Gordan, (22) Houston, Andrewes and Horder (17). Gordon has selected saccharose, lactose, raffinose, inulin, salicin, coniferin and mannite taking into consideration also the reactions in neutral red broth and litmus milk. The reactions with these media constitute the "Gordon's metabolic tests" for the streptococci. Over twelve hundred strains have now been submitted to Gordon's tests. Andrewes and Horder have summarized the results obtained and conclude that while in themselves the chemical tests are too arbitrary to form a basis for a systematic classification yet taken in conjunction with other characters "they afford a clue to the nature of any given streptococcus which is invaluable." As a result of these observations they have roughly classified streptococci into seven groups. "We venture to believe that some such conception of the streptococci as we have set forth is preferable to the idea that they are all of a kind or that they present a hopeless chaos."

Gordon's Metabolic Tests.

Types of Streptococci.	Clot in milk.	Reduction of Neutral Red.	Saccharose.	Lactose.	Raffinose.	Inulin.	Salicin.	Coniferin.	Mannite.	Gelatin at 20 C.	Morphology.	Pathogenicity for mice.
Equinus.	-	-	+	-	-	-	+	+	-	-	Medius	-
Mitis.	-	-|+	+	+	-	-	+	+	-	+	Brevis	-
Pyogenes.	-	-	+	+	-	-	+	-	-	+	Longus	+
Salivarius	+	+	+	+	+	+	-	-	-	+	Brevis	-
Anginosus.	+	+	+	+	+	-	-	-	-	+	Longus	+
Fecalis.	+	+	+	+	-	-	+	+	+	+	Brevis	-

This classification has been attacked on several grounds. Walker (23) asserted that fermentation reactions were not constant in any strain, but vary from time to time. Buerger (24) found that such a grouping does not correspond to the type of pathogenicity of the strain. Winslow (25) and others in this country maintained that the quantitative determination of the acid produced was essential to an accurate study of fermentations. However after very extensive work on saprophytic types they arrive at no very definite results. Helman (30) in an extensive study of many strains of streptococci used Andrewes and Herder's classification but with many modifications. More than 2400 streptococcus strains have been adapted to this scheme. His plan of classification is herewith given.

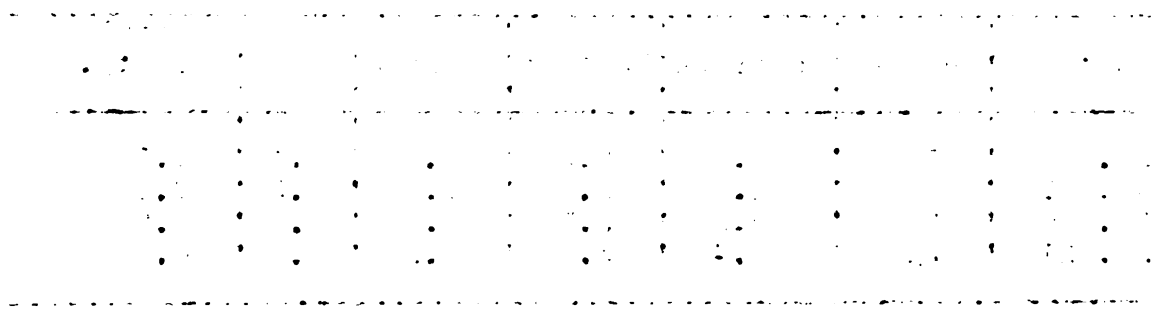

STREPTOCCI
Gram-positive cocci in chains, no capsules.
HEMOLYSIS
+
-
Lactose
+
-
Mannit
+
-
Salicin
Str. Hemolyticus
Str. Hemolyticus
Str. Pyogenes
Str. Anginosos
Str. Hemolyticus ii
Str. Hemolyticus iii
Str. Equi
Str. Subacidos
Str. Fecalis
Str. Non-Hemolyticos i
Str. Mitis
Str. Salivanios
Str. Non-Hemolyticus ii
Str. Non-Hemolyticus iii
Str. Equinos
Str. Ignavus

A summary of the literature reveals the fact that the classification of streptococci according to fermentation reactions alone has led to the establishment of a bewildering number of types. Andrewes and Horder group all these types under six heads to which they have given specific names. They showed a definite relation between their grouping by cultural tests and the pathogenicity and source of the organisms.

J. Broadhurst (26) has shown that in the fermentation of various carbohydrates by streptococci that a higher degree of acidity is reached using meat infusion broth than when meat extract broth is used. Accordingly meat infusion has always been used in prepafing the media for making the fermentation tests reported in this study. Table VII shows that a much higher degree of acidity is reached when two per cent peptone (Witte's) is used than when only one per cent is used. The more vigorous growth with two per cent of peptone present probably makes possible a greater utilization of energy derived from the splitting of the carbohydrate.

Table VII.

Culture	Peptone	Dextrose	Lactose	Mannite	Plain	Initial Acidity.
No. 30	1%	3.8	2.3	1.6	0.4	0.2
No. 21	1%	2.4	2.1	1.0	0.3	0.2
No. 30	2%	5.5	3.7	3.0	0.7	0.2
No. 21	2%	3.4	3.0	2.2	0.3	0.2



The sugar broth tubes were inoculated with one drop of a twenty-four hour broth culture from a pipet. This method was quite convenient and gave uniform results. Titrations were made after twenty-four hours incubation at 37°C. But very little acid is formed after the twenty-four hour period as is shown in Table VIII below:

Table VIII.

Culture	Carbohydrate.	24 hours.	48 hours.	72 hours.
No. 30.	Lactose	2.3	2.3	2.5
No. 30.	Dextrose	3.8	4.6	5.0
No. 21.	Lactose	2.1	2.4	2.6
No. 21.	Dextrose	2.4	2.9	3.7

Therefore for the remainder of the tests, a medium containing two per cent peptone (Witte's), 0.5 per cent sodium chloride adjusted to a neutral reaction was used. To this was added one per cent of the fermentable substances used, which were of the following brands:

Lactose, J. T. Baker Chemical Co.

Salicin, Eimer and Amand.

Rafinose, Kahlbaum.

Mannite, Merck.

Inulin, Kahlbaum.

It was found that saccharose was fermented by all strains hence this carbohydrate has not been used in the test. Dextrose, levulose, galactose and dextrin are of no value in classification as Buerger (24), Winslow and Palmer (27), and Artz (28) have found that these carbohydrates are fermented by all streptococci. On the other hand none have been found to ferment adonite or dulcite. Tests for

the reduction of neutral red was not undertaken. As reported by Buerger (29), and Hppkins and Lang (13) this test appears to be very indefinite because concordant results are not obtained in repeating the test.

In the titration for the amount of acid produced, five c.c. of the contents was titrated against H/20 NaOH using the first pink of phenolphthalein as the end reaction A correction was made by inoculating a tube of plain broth and noting the amount of acid formed, during a like period of incubation. This plan was adopted to avoid the use of a colon-free sugar broth, partly because some streptococci grow feebly in a colon broth and also to exclude possible acid formation from non-carbohydrate constituents.

Following the suggestion of Hopkins and Lang (13) I have considered as fermenting organisms only those which have produced over .8 per cent of acidity while those which produced only .8 per cent or less I have considered as non-fermenting types, the initial acidity having been deducted from the titre. In Table IX is given a record of fermentation reactions of the thirty eight strains with five different test sugars. These reactions have been noted over a period of five months.

Table IX

FERMENTATION OF CARBOHYDRATES BY STREPTOCOCCI.

	Lactose				Raffinose				Salicin				Mannite				Inulin			
	March 20	May 3	June 8	August 15	March 20	May 3	June 8	August 15	March 20	May 3	June 8	August 15	March 20	May 3	June 8	August 15	March 20	May 3	June 8	August 15
1 Udder	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
2	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
3	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
4	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
5	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
6	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
7	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
8	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
9	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
11	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
12	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
13	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
14	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
15	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
16	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
17	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
18 Sore throat	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
19	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
20	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
21	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
22	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
23	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
24	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
25	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

		Lactose				Raffinose				Salicin				Mannit				Inulin			
		March 20	May 3	June 8	August 15	March 20	May 3	June 8	August 15	March 20	May 3	June 8	August 15	March 20	May 3	June 8	August 15	March 20	May 3	June 8	August 15
26	Sore throat	-	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-
27	Normal "	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
28	" "	+	+	+	+	+	+	+	-	+	+	+	+	-	-	-	-	-	-	-	-
29	Hemaliticus	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-
30	Pyogenes	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-
31	Sputum	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-
32	Stock culture	+	+	+	+	0	0	-	-	0	0	+	+	0	0	-	-	0	0	-	-
33	Pyogenes	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	+	+	+	+
34	Endocarditis	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-
35	Hemalyticus	+	+	+	+	0	0	-	-	0	0	+	+	0	0	-	-	0	0	-	-
36	Metachramotos	+	+	+	+	0	0	+	-	0	0	-	-	0	0	-	-	0	0	-	-
37	Milk	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	+	-	-	-	-
38	Peritonitis	+	+	+	+	-	-	-	-	+	+	+	+	-	-	-	-	-	-	-	-

Table IX well sets forth the remarkable constancy of the fermentation reactions. Even after five months of artificial cultivation all but seven strains continue to give the same reactions. This is in quite direct opposition to the report of Bergey (29) who says: "Unfortunately, the streptococci are very easily affected in their fermentative powers so that it is not an easy matter to obtain concordant results upon repeating the tests after the organisms have been grown in artificial media for some time."

Homologous and Heterologous Agglutination and Complement Fixation Reactions upon Animal Inoculation.

Ever since it has been known that immune sera often agglutinate the bacteria concerned in their production, constant efforts have been made by investigators to determine the value of this reaction in the case of various bacteria which closely resemble each other. Streptococci have been included among the bacteria thus studied and certain facts have been learned regarding the power of the sera to cause their agglutination. Significant antigenic dissimilarities among closely related streptococci have been determined by the agglutination test.

The relationship of streptococci to disease as well as the relationship of different streptococci to one another was at first based upon the results of the agglutination reactions. In 1902 Aronson(31) made very complete observations upon the agglutination action of the sera of horses which had been immunized. The streptococci employed in the tests and the immunization were rendered

highly virulent by multiple passage through mice. He found that the serum from a horse treated with a certain streptococcus agglutinated other cultures as well as the homologous one. His observations point to a close relation - ship between all streptococci. Andrewes and Horder found agglutination reactions "troublesome and disappointing" but attempts were soon directed toward a correlation of agglutination and fermentation reactions. Floyd and Wolbach (32) concluded that fermentation reactions could be used to separate streptococci into large groups, while agglutination reactions merely emphasized the individuality of the members. Kligler (33) on the other hand found that the agglutination reactions ran strikingly parallal with fermentation reactions. Though the agglutination reaction was not found to separate the streptococci into large group he says: "the agglutination test tends to show that a division of the streptococci on the basis of hemolysis is not warranted, whereas, a separation according to the fermentation reactions appears to coincide more closely with their natural relationship." Swift and Thro (34) report that the agglutination test is specific for streptococci but not specific for individual strains.

Seven years ago Besredka applied the complement-fixation reaction to the study of horses which had been immunized to streptococci and found that the serum of such horses gave a fixation of complement. The various strains were specific. Fox and Mallein (35) studied scarlet fever patients. The sera from ten of the twelve patients with scarlet fever gave positive reactions, with nine controls,

among which were four of erysipelas and one of puerperal sepsis, which gave negative results.

From the foregoing it will be seen that the question of classification is unsettled when undertaken from an immunological standpoint.

Naturally of primary importance in the technic of the agglutination test was the preparation of a satisfactory antigen. Attempts to prepare an antigen by growing the organisms on solid media and subsequently washing off with salt solution were not successful. Growth of these organisms on most solid media is scanty. All agglutination antigens reported inthis study were prepared as outlined by Hiss (36). Erlenmeyer flasks containing 250 c.c. of a 2 per cent peptone meat infusion broth with 1 per cent of dextrose and 1 per cent of calcium carbonate were sterilized by the Tyndall method and subsequently inoculated. These flasks were incubated at 37°C. for four days and twice each day the flasks were shaken. The shaking serves the purpose of breaking up the longer chains so after the first twenty four hours the cultures becomes uniformly turbid. At the end of the incubation period the flasks were shaken and then set aside for one hour during which time the calcium carbonate settles to the bottom of the flask. The turbid broth culture was then pipeted off with a large pipet attached to an aspirator. The antigen was then diluted with sterile salt solution until in density it closely compared with tube No. 2 of McFarland's nepholometer (37) and then stored in bottles with .5 per cent of formalin added as a preservative.

It is a well known fact that formaldehyde even in very small amounts will exert a peculiar action on proteid material, hardening it or otherwise altering its chemical and physical properties. In order to determine the amount of formalin which would interfere with agglutination a known positive serum was drawn and added to a series of tubes with antigen containing various amounts of formalin. The formalin was allowed to remain in contact with the antigen for a period of thirty minutes before placing in the tubes for the agglutination test. Inasmuch, as the action of the formaldehyde might be more pronounced after a longer exposure, ~~another series of tubes was run after a longer exposure~~, another series of tubes was run after twenty four hours exposure to the formalin. A third series was also run after several days exposure at ice box temperature. Results of the three series are given in Table X.

Table X.

Antigen No.	Serum No.	Per cent of formalin present.	Length of time formalin present.	Agglutination. 1:50	1:100	1:200	1:330	1:500	1:650
9	165	0.5	30 min.	+	+	+	+	+	-
9	165	1.0	30 min.	+	+	+	+	+	-
9	165	2.0	30 min.	+	+	+	+	-	-
9	165	3.0	30 min.	+	+	+	P	-	-
9	165	4.0	30 min.	+	+	+	-	-	-
9	165	5.0	30 min.	+	P	-	-	-	-
9	165	0.5	24 hrs.	+	+	+	+	+	-
9	165	1.0	24 hrs.	+	+	+	+	P	-
9	165	2.0	24 h4w.	+	+	+	P	-	-
9	165	3.0	24 hrs.	+	+	+	-	-	-
9	165	4.0	24 hrs.	+	+	+	-	-	-
9	165	5.0	24 hrs.	+	+	P	-	-	-
9	165	0.5	7 days.	+	+	+	+	+	-
9	165	1.0	7 days.	+	+	+	+	P	-
9	165	2.0	7 days.	+	+	+	+	P	-
9	165	3.0	7 days.	+	+	+	P	-	-
9	165	4.0	7 days.	+	+	P	-	-	-
9	165	5.0	7 days.	+	+	P	-	-	-

Note: + Agglutination.
P Partial agglutination.
- No agglutination.

From the foregoing table it will be noted that 1 per cent of formalin does not interfere with the agglutination of an antigen by its specific serum. Two per cent of formalin appears to have a slight inhibitory effect But little difference is noted in subjecting the antigen to the formalin for one-half Hour or for one week. The inhibitory effect does not seem to be cumulative.

In running the agglutination test five dilutions were used. Two cubiccentimeters of the antigen were place in each of the five tubes and 0.5 cc. of the serum was added te 4.5 c.c. of salt solution. From this 1 to 10 serum mixture 0.4 c.c., 0.2 c.c., 0.1 c.c., 0.04 c.c., 0.02 c.c. were added to the respective tubes which in the order listed give approximate dilutions of one te fifty, one hundred, two hundred, five hundred and one thousand.

The tubes with control tubes of antigen only were incubated at 37° C. for at least twelve hours, when results were read. Complete agglutination is marked by the formation of clumps or aggregates of bacteria which soon settle to the bottom of the tube leaving the supernatent liquid clear. There is however in all tubes a primary deposit of particle of the calcium carbonate which is not to be mistaken for agglutination, when comparison is made with the control.

In making the complement fixation test, the simplest kind of an antigen was used. Flasks containing 200 c.c. of dextrose broth were inoculated and incubated for forty-eight hours at the end of which time the culture was placed in a large centrifuge tube and centrifuged for fifteen minutes. The large mass of organisms in the bottom of the tube was washed three times with phenolized (0.5 per cent) salt (0.85 per) cent solution. A sufficient amount of the solution was added after the last washing to bring the turbidity of the suspension to compare with tube No. 5 of McFarland's nepholometer (37). Antigen so prepared was found to have a very satisfactory "antigenic titre" in the presence of a known immune serum and not an undue anticomplementary titre.

In the routine procedure of the complement fixation tests, complement was obtained from guinea pig's blood. Blood was taken from the pig's, <u>paracentesis cardii</u>, by inserting a small hypodermic needle into the left chamber of the heart. The clear serum was removed and diluted one to four with physiological salt solution.

Varying amounts of the diluted complement were added to constant amounts of hemolytic amboceptor (1 to 100) and 0.5 c.c. of a 2.5 per cent suspension of sheep's blood cells. In running the test proper, twice the titre or two units of complement were used as the dose.

Hemolytic amboceptor was obtained by injecting a healthy normal rabbit with washed sheep cells as follows: April 27, one c.c. of cells, April 29, two c.c. of cells and May 2, two c.c. of cells were injected intravenously.

On May 4th., or two days after the last injection, the animal was bled. At that timme the serum, diluted 1 to 100 effected complete hemolysis of 0.5 c.c. of a 2.5 per cent suspension of sheep cells in as small amounts as 0.02 c.c. in presence of complement. This was considered to be a very strong serum considering the short period of immunization and the close proximity of bleeding. On two or three different occasions within the next month 30 c.c. of blood was taken from the heart each time and replaced with physiological salt solution, given intracardially. Notwithstanding the successive bleedings the serum maintained its relatively high titre.

Preparatory to running the preinjection tests for non-specific reaction of the rabbit sera, the antigens were each titrated to determine their anticomplementary action. Varying doses of antigen were added to two units of complement and kept in the water bath for 30 minutes at 37°C., subsequent to which two units of hemolytic amboceptor and 0.5 c.c. of a 2.5 per cent suspension of sheep cells were added. One half the smallest amount of antigen giving

inhibition of hemolysis was taken as the dosage. When positive serum was available five times the smallest amount giving complete inhibition in the presence of a positive serum was used as the dose, provided of course that the amount did not interfere with hemolysis in the absence of a positive serum.

In making a large number of tests, which was often necessary, the labor involved in pipeting of the materials was minimized by adding the proper amount of complement (1 to 4) to a known amount of salt solution so that the exact dose of complement was contained in 1.5 c.c. of the salt solution, which was used in all tubes for dilution. Likewise, the correct amount of hemolytic amboceptor (1 to 100) was added to a known amount of the sheep cell 2.5 per cent suspension so that 0.5 c.c. of the suspension contained the proper dose of the amboceptor. The combining of the salt solution and complement and of the hemolytic amboceptor and sheep cell suspension reduces to a considerable extent the labor involved in pipeting. Titration of the complement dilution made with the hemolytic amboceptor-sheep cell mixture after being mixed for as long as eight hours compares with that made before mixing the materials, so that uniformity is assured in running successive tests.

Healthy, normal, average-sized rabbits were selected for the immunization. Pre-injection tests, both for agglutinins and complement binding substances were made. Suspensions of organisms for inoculation were prepared by centrifuging one twenty-four-hour dextrose broth culture of each organism, removing supernatent broth and suspending

each bacterial mass in five c.c. of sterile salt solution.

Four inoculations were given intraperitoneally as outlined below:

June 16, 3 c.c. killed by heating to 60°C. for 30 minutes.

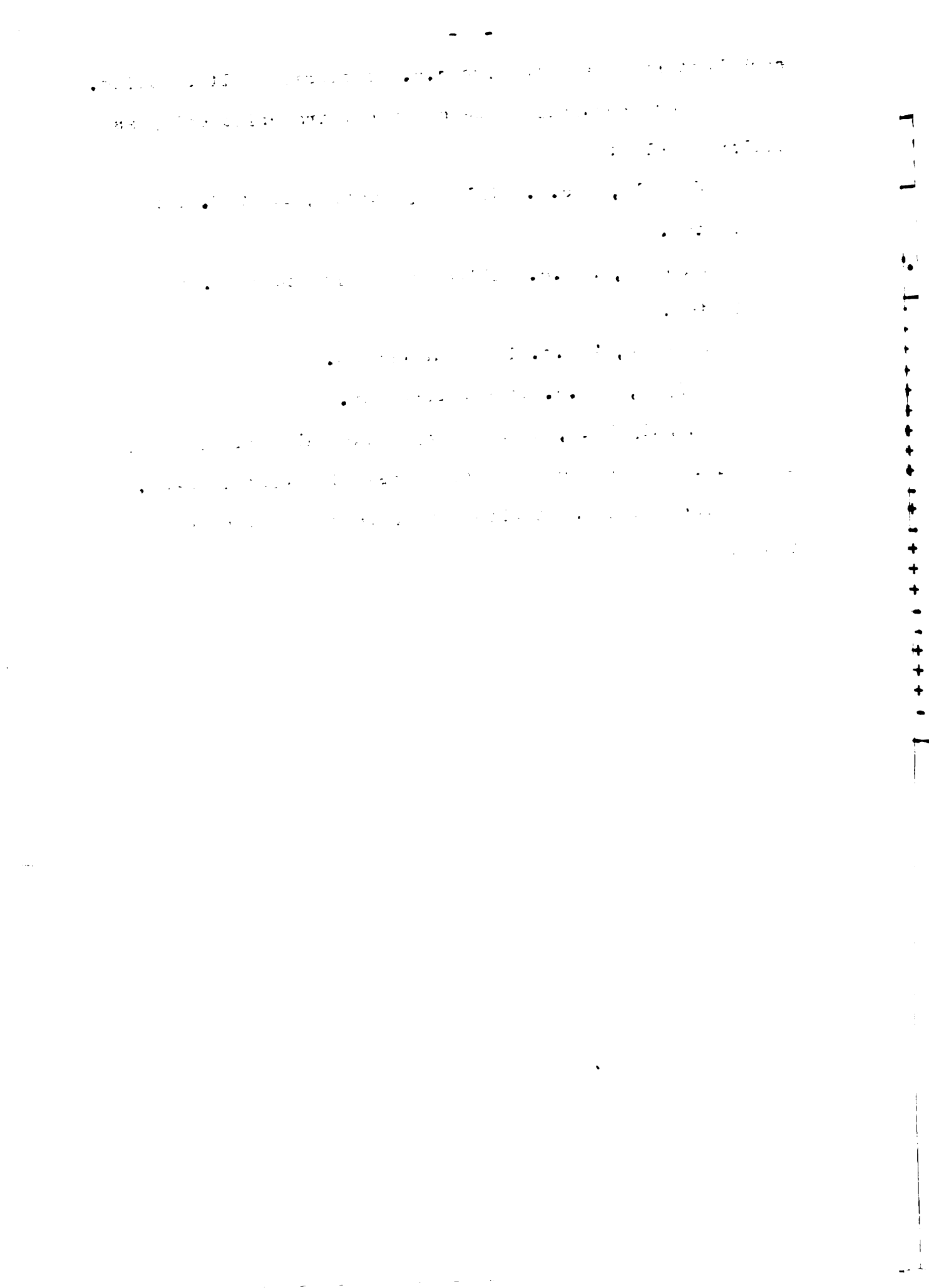
June 23, 5 c.c. killed by heating to 60°C. for 30 minutes.

June 30, 2 c.c. live suspension.

July 7, 5 c.c. live suspension.

On July 16th, or nine days after the last injection the animals were bled and serum tests immediately made.

The following tables give a record of the serum tests:



19	Sore throat Aggl.
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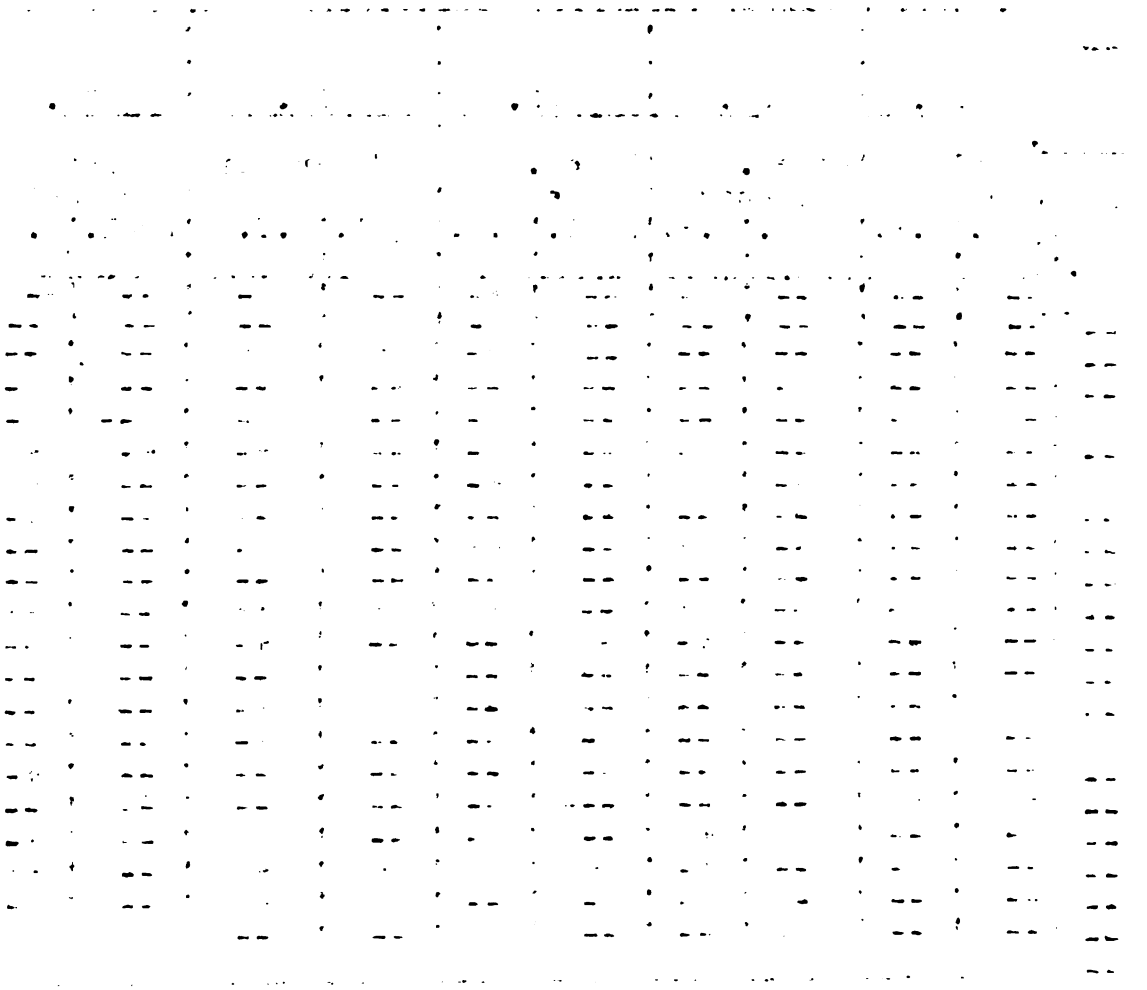

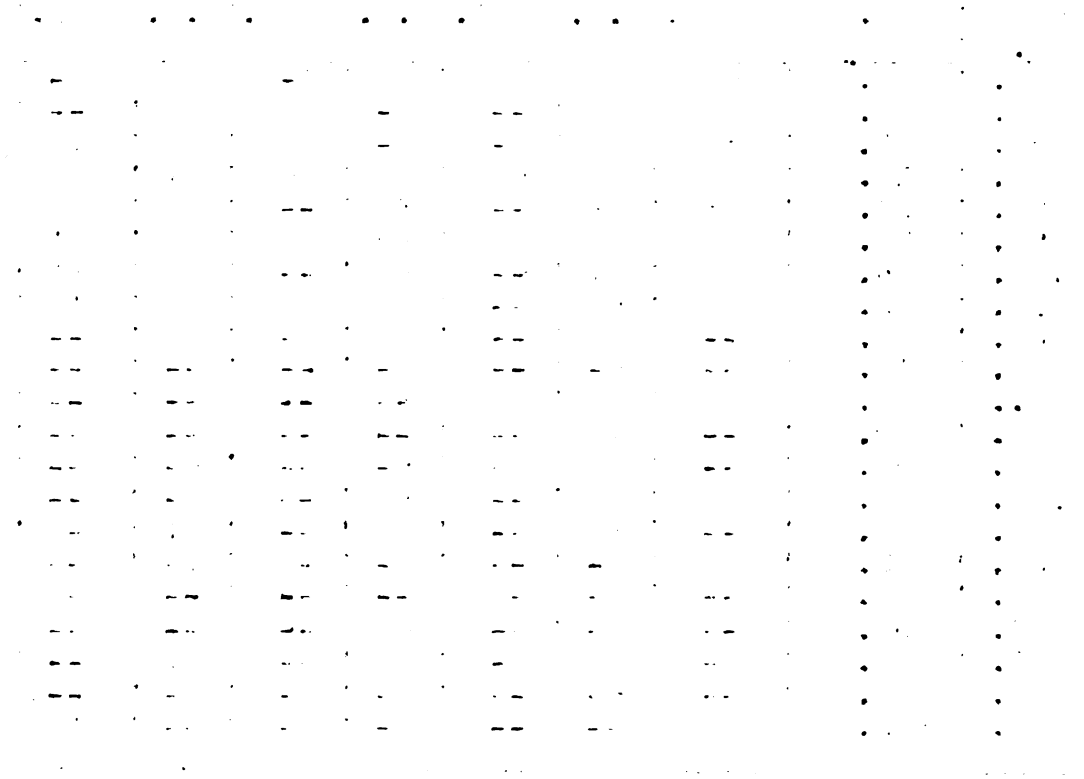

Table XI shews but little strain specificity or of group specificity. From a standpoint of discovering a satisfactory technic the results are very encouraging but after little or no hope of showing the specificity of an antistreptococcus serum to its own antigen. These results are much in accord withthe reports of Brown and Hitchens who, after an extensive study of the antigenic properties of various streptococci, say in part: "It was very surprising to note how some immune sera gave cross-fixation with a large number of antigens. Out results did not seem to show that the complement-fixation method was of much service in classification."

Summary.

From the diseased udders of cows, hemolyzing and non-hemolyzing streptococci have been isolated. These organisms through their fermentative activities are to be classified as pyogenes and mitis. None are to be considered as belonging to the anginosus group which is considered as the etiologic factor of sore-throat.

Future research is necessary to demonstrate the correlation between exaltation in virulence, as by animal passage, and that of fermentative properties together with hemolytic determinations. In the mind of the writer this should form the basis for very valuable research in determining the nature of that elusive organism, the streptococcus.

Agglutination and complement-fixation reactions failed to demonstrate either strain or group specificity.

In concluding I wish to acknowledge my indebtedness to Professor L. H. Cooledge and to Dr. Ward Giltner for suggestions and assistance received during this investigation.

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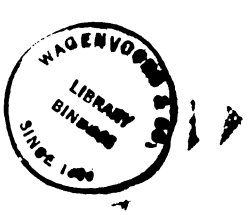
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