A Study of Hemolytic Streptococci as Found in the Tropical Island of Puerto Rico*

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INTRODUCTION

THERE is no organism among the bacteria that has aroused more interest than the streptococcus. Its manifold activities, its complicated antigenic structure and the variability of its behavior when placed in different environments have rendered this morphological type a difficult one to study. However, its ability to produce in the human body a variety of pathological conditions such as scarlet fever, puerperal sepsis, erysipelas, septic sore throat and tonsillitis, and its alleged connection in the etiology of rheumatic fever, have greatly stimulated investigators in all parts of the world to study the streptococcus. The hemolytic strains, because of their greater virulence and association with a larger number of human diseases, have been the subject of more intensive investigation. Most of these investigations, however, have been made in countries within the temperate zone or with strains originally isolated from patients in this environment. The incidence of hemolytic streptococci is greater and is associated with more serious infections here than in semitropical or tropical regions.

The throat is the main reservoir of hemolytic streptococci in the human body. In a place like New York City, situated well within the north temperate zone, the incidence of beta hemolytic streptococci in the throats of healthy individuals is high, although there is a marked seasonal variation.[15] On the other hand, in places like the island of Puerto Rico or the nearby island of Saint John,* both situated well within the tropics, the number of normal persons harboring hemolytic streptococci in their throats is low and no con-

* Milam and Smillie (59) found that on the well isolated tropical island of Saint John "only three per cent of the people harbored true *St. pyogenes*, and when found the organisms were few in number," and they expressed their belief "that *St. pyogenes* is essentially an organism of civilization and industrial overcrowding." The results of our previous studies 69 and of the present investigation, as will be seen later, show that the incidence of true beta hemolytic streptococci in the throat of normal individuals in the city of San Juan, the capital of Puerto Rico, is about the same as found by Milam and Smillie in Saint John. The city of San Juan has nearly one hundred and fifty thousand inhabitants, the majority of which live in overcrowded settlements.

spicuous variations are observed throughout the year[61, 59, 69]. Thus it is apparent from the work of Coburn[15] and others[61, 59, 69, 74, 20] that the incidence of this organism in the throats of normal people diminishes upon approaching the tropics.

It is not less true, however, that in Puerto Rico sore throats and diseased tonsils are common, and that true beta hemolytic streptococci are frequently isolated in large numbers from these sources. Furthermore, the association of this organism with the first attacks of recurrent lymphangitis, a condition very common in Puerto Rico[62, 81, 82] and in other tropical countries[36], is remarkable. It is so much so, that provided the proper technique is employed, streptococci can be recovered in pure culture during the acute attack in practically every case of lymphangitis where a lesion is present. It is also a fact that in this Island hemolytic streptococci are frequently obtained from such varied sources as abscesses, otitis media, mastoiditis, osteomyelitis, and are very common in a variety of skin conditions[107, 1, 2] affecting especially the lower extremities of children. Thus in certain pathological conditions in Puerto Rico the association of beta hemolytic streptococci is marked even though the incidence in the normal throat is low.

In view of what has been said it is puzzling to find scarlet fever and rheumatic fever, diseases which are so prevalent in New York City, to be relatively rare in Puerto Rico, and when they occur the course is quite mild. The following explanations for the aforementioned discrepancies suggest themselves: 1. The biological activities of the streptococcus may be affected by the difference in environment. 2. The physiology of the host may be different. With the idea of throwing some light on the first of these suggestions the present work has been undertaken.

Certain attempts by others have been made in this direction. In the year 1931 Grace and Grace[36] studied "the relation between cultural and serological characters of certain strains of hemolytic streptococci" and the "bacteriology of streptococcal strains" isolated by them from different sources in Georgetown, British Guiana. A comparison of some of these strains with others isolated in temperate climates was attempted. Teiger and Seegal in 1936[84] made a "comparative study of streptococci isolated from throats of residents of New York and New Orleans." In the same year Davis and Guzdar[20] studied 78 strains isolated from the throats of normal Hong Kong Chinese.

We have studied 191 strains of beta hemolytic streptococci isolated

in Puerto Rico in considerable detail. The results obtained have been compared with those of other investigators working with strains from similar sources isolated in temperate climates. As a rule most of the tests were run concurrently on each strain. In the majority of cases the strains were tested shortly after isolation and in many instances the tests were repeated at regular intervals thereafter.

Among the cultures investigated, twenty-two strains isolated from the throats of apparently normal monkeys have been included. Twenty-one of these were cultured from monkeys which were recent arrivals in Puerto Rico. The results of the study of these strains are reported here due to their similarity with those obtained with strains isolated from human throats.

COLLECTION OF MATERIAL AND METHODS OF ISOLATION

Throat specimens. The surfaces of both tonsils and the pillars were rubbed with sterile absorbent cotton swabs stored in glass tubes. When seedings were not made immediately they were placed in the ice box and cultured as soon as possible, always within six hours after collecting the specimen.

One half c.c. of sterile defibrinated rabbit blood was placed in each of three Petri dishes. Three tubes containing from fifteen to eighteen c.c. of plain beef extract agar[102] were melted and cooled to 45–50° C. The swab was immersed in the melted agar in the first tube and thoroughly wetted. Then it was raised above the surface of the liquid and gently pressed against the wall of the tube so as to squeeze out the material into the medium. This was repeated two or three times in rapid succession and the swab discarded. With the aid of a sterile 1 c.c. pipette the upper portion (the upper ½ inch) of the agar was thoroughly mixed with the material inoculated. The tip of the pipette was drawn above the surface of the liquid and any agar inside the pipette discharged. Then with this same pipette, from 0.05 c.c. to 0.1 c.c. (depending on the amount of material on the swab) of the inoculated medium in tube one (upper portion) was transferred to a second tube of agar. Using the same pipette and following the same technique, 0.05 to 0.1 c.c. was again transferred from the second to a third tube of melted agar. The content of each tube was then poured over the blood in three corresponding Petri dishes. The dishes were promptly stacked and the media thoroughly mixed. After solidification of the medium the plates were inverted and incubated aerobically at 37° C. for twenty-four hours, at which

time they were examined for the presence of deep streptococcus colonies showing true beta hemolysis.* †

A small agar block containing only one well isolated colony was cut out with a sterile sharp spatula and transferred to a tube of glucose neopeptone broth, it was macerated with the spatula against the wall of the tube and incubated for 20 hours. The surface of the culture was touched with a straight wire and a poured blood agar plate prepared. After 20 hours incubation the process of purification was repeated as before. This process was carried out three times in order to obtain pure cultures. The growth in the last broth culture was transplanted to the surface of a five per cent blood agar slant and stored in the ice box. Transplants from this stock culture were employed for the studies reported in this work.

Tonsils. The tonsils were placed by the surgeons in sterile Petri dishes or specimen bottles and brought to the laboratory as soon as possible.‡ The openings of the crypts were carefully seared with a chromel wire after which the loop was introduced deep into the crypts and streak cultures made on blood agar. Then the surface of the tonsil (avoiding the crypts) was seared, an incision was made with a sterile knife, the inoculating loop subsequently introduced through the incision into the deep tissue and the material obtained was cultured. If streptococci were present pure cultures were obtained, as mentioned previously in the case of those from the throat, before they were finally transplanted to blood agar slants and put in stock.

Small ulcers, pustules and abscesses. In taking cultures from small ulcers, they were thoroughly cleaned with sterile cotton or gauze, the tissue was gently squeezed and the exuding fluid streaked on a blood agar plate.

In the case of pustules the pus was cultured first, then the scab was removed and the pus was cleaned off thoroughly with a sterile gauze, the tissue was gently squeezed and the serous fluid oozing from the lesion was cultured. In many instances (from pustules, and

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* By "true beta hemolysis" is here meant that appearing in 24 hours or before, at 37° C. The hemolyzed zone is clear and free from blood cells when examined microscopically. It is two or more mm. in diameter. It increases somewhat in size when left in the incubator for 48 hours but remains the same diameter when the plates are kept in the ice box.

† The presence of hemolytic hemophilic Gram-negative bacilli is sometimes confusing. To obviate this a medium in which sheep blood is used has been recommended 52. A Gram stain will also eliminate this difficulty.

‡ In this connection we are indebted to Doctor Muñoz McCormick, of the medical staff of the Mimiya Clinic in Santurce, and to Miss Cecilia Benítez, of the School of Tropical Medicine, for their kind cooperation.

from small ulcers in some cases of lymphangitis) cultures from the pus gave a pure culture of staphylococci or a mixed culture of both streptococci and staphylococci. However, cultures from the exuding fluid, after thorough cleaning of the pus, yielded hemolytic streptococci in pure culture. *The failure to clean off the pus in these cases and take cultures of the exuding fluid has resulted in many instances in the cultivation of staphylococcus from a lesion in which hemolytic streptococci were the organisms really concerned.*

In the examination of pus from abscesses a Gram stain was made as a preliminary step. The material was diluted with saline, when many organisms were seen in direct smear. A loopful of the suspension was placed on a blood agar plate and spread all over the surface with a sterile swab.

SOURCE AND INCIDENCE OF THE HEMOLYTIC STREPTOCOCCI STUDIED

*Normal throats. Selected cases. Single cultures.** In previous work done by the author[69] single cultures were taken from 200 persons whose throats had been free from symptoms for at least two years and appeared normal at the time that they were swabbed. The beta hemolytic streptococcus was obtained in 4 per cent of these cases.

Throats of general population. Unselected cases. Single cultures. These were from male workers in camps situated in the central part of the Island and from male workers in dairy farms near San Juan. Four hundred and sixteen cases were studied. These may be grouped as follows:

Three hundred and seventy were free from throat symptoms although many of these had hypertrophic or cryptic tonsils in some instances accompanied by a hyperemic pharynx. The incidence of beta hemolytic streptococci was 6 per cent.

Forty-three persons gave a history of recent throat trouble but were asymptomatic at the time cultures were taken. From these, beta hemolytic streptococci were recovered in approximately 30 per cent.

Three individuals actually had a sore throat. Beta hemolytic streptococci were obtained from two of them.

If these 416 are typical of the general population, irrespective of the condition of the throat or of any previous history of throat trouble, the distribution of hemolytic streptococci would be 9 per cent.

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* Only one culture taken from each case.

*Clinic cases. Unselected. Serial cultures.** The great majority of these were patients reporting to the lymphangitis clinic of the University Hospital at San Juan. Sixty-five were studied.** The time during which the throat of the same person was examined varied from one to ten months and the number of cultures taken from the same individual, from two to sixteen. A total of 305 cultures were made.

In thirteen (20 per cent) instances beta hemolytic streptococci were obtained when the initial culture was taken. Eleven of these thirteen cases either gave a history of sore throat or had some other abnormality (chronic tonsillitis, hyperemic pharynx, etc.). Two had normal throats.

By repeated swabbing, six more positive cultures were obtained. Two of these were from normal throats. This increased the incidence from 20 per cent in the case of single cultures to 29 per cent when serial cultures were used.

In some individuals the streptococci disappeared and reappeared at irregular intervals. These fluctuations were not necessarily associated with the appearance or the exacerbation of throat symptoms.

It is of interest to note the difference between the incidence of hemolytic streptococci in the throat of the general population (9 per cent) and in the throat of cases suffering from recurrent lymphangitis (20 per cent). Whether or not there exists any relationship between the presence of beta hemolytic streptococci in the throat or in the tonsils and the acute attack of lymphangitis we are at present unable to say. This is now being studied.

It is difficult to evaluate the findings of different investigators concerning the incidence of true beta hemolytic streptococci in the normal throat. Some workers do not specify the place where the studies have been made, others do not state the time of the year and whether they have used single or serial cultures. Furthermore, it is difficult to determine what in the opinion of the different authors is a normal throat. It is obvious that under these circumstances the figures found in the literature will exhibit wide variations. A number of these references has been brought together in table I.

Throats of apparently normal primates. The apparently normal throats of six gibbons, one spider monkey, and eleven rhesus monkeys that had been in the Island for one year or more were cultured

* Cultures taken from the same person at frequent intervals.

** We are indebted to Dr. Juan A. Pons for his kind cooperation in obtaining material for examination from these cases.

and beta hemolytic streptococci recovered in one instance* (5.5 per cent).

The throats of 172 rhesus monkeys brought to Puerto Rico from Calcutta were also studied.† Cultures were taken over the period from November 20 to December 12, 1938. From these animals beta hemolytic streptococci were recovered and studied in detail in 22 cases (12.7 per cent). There were some instances, however, in which suspicious colonies were present, but the streptococci could not be identified with certainty due to the overgrowth of *B. proteus* or spore forming aerobes. Had it not been for the contamination the incidence of hemolytic streptococci in the throats of these monkeys would probably have been somewhat higher.

Seegal, Heller and Jablanowitz[73] studied the flora of 49 rhesus monkeys in New York City and cultured beta hemolytic streptococci from 28 of them (57 per cent).

Dochez, Shibley and Mills[23] in connection with their experimental studies of the common cold found the incidence of hemolytic streptococci in the nose and throat of apes to be 1.2 per cent and 45 per cent respectively.

Pathological throats. The swabs were taken and sent to us by a throat specialist. From a total of 19 cases with a clinical diagnosis of acute tonsillitis, hemolytic streptococci were obtained from fifteen (80 per cent). In ten cases with a history of recurrent tonsillitis but from which cultures were taken from one week to one month after the acute attack beta hemolytic streptococci were obtained in only three (30 per cent).

Tonsils (operated). The excised tonsils of 25 patients were examined. Cultures were made from the crypts and from the inside. Beta hemolytic streptococci were cultured in seven cases (28 per cent). In work previously done by the author[69] one hundred pairs of tonsils were examined and beta hemolytic streptococci cultured in 33 per cent.

Vagina. Aerobic cultures taken from the vagina of 25 patients suffering from different conditions were negative for hemolytic

* Strain P. R. Mo. 1 from spider monkey.

† We are indebted to Dr. C. R. Carpenter for kindly furnishing us with the following information: These monkeys came, on the average, from about 300 miles inland from Calcutta. The period of time they remained in Calcutta awaiting shipment varied with different animals from a few days to three weeks. The shipment left Calcutta September 30, 1938. The ports touched en route in order were: Colombo, Ceylon, one day. Food taken on. Capetown, South Africa, one day. Purchased fruits and vegetables. Boston, Mass., two days. New York City. Here the animals were trans-shipped during which time they remained on the dock for 48 hours.

streptococci in every instance. Two of these women harbored a beta hemolytic streptococcus in their throats. One had a normal throat. The other had a sore throat.

Lymphangitis. Cultures taken from pustules or small ulcers of the affected limb, during the acute attack, in 28 cases of lymphangitis revealed the presence of hemolytic streptococci in every instance. The association of this organism with the initial attack of this condition is striking.

Bovine mastitis. Material from the udders of 130 cows with mastitis was studied. Beta hemolytic streptococci of the type usually encountered in human infections (group A) and in the normal human throat (groups C and G) were not found. *Streptococcus agalactiae* (group B) was the organism isolated from more than 90 per cent of the cases. This is in accordance with the observations of others working in temperate climates.

Strains isolated from other sources (otitis media, puerperal sepsis, abscesses, osteomyelitis, etc.) are also included in the present investigation. We do not possess accurate information about the incidence of the beta hemolytic streptococcus in these conditions in Puerto Rico.

Table II shows the distribution among the different sources of the strains studied in this investigation.

GROWTH CHARACTERISTICS, MORPHOLOGY AND STAINING

A detailed study of the colony types, the growth characteristics in liquid media, the variation in morphology of the bacteria and the possible relationship of this appearance with the biological properties of the streptococci studied has not been attempted. This would have constituted a major problem in itself. However, some observations made during the course of this work will be recorded here.

Appearance of colonies on blood agar. The morphology of 24 to 36 hour old, well isolated colonies on streak blood agar plates incubated at 37° C. was observed soon after isolation.

Four different types of colonies were encountered:

1. The first type was smooth, soft, easily emulsified, usually low conical but in some instances low convex. Sometimes the colonies were definitely raised and either dome shaped or high conical. Occasionally they had the appearance of a truncated cone with a small flat round area at the top. The edges of the colonies were usually even, but occasionally they were slightly wavy. They varied in size

when well isolated from ½ mm. to about 2 mm. in diameter. Under the low power of the microscope and by transmitted artificial light, a round black dot was seen at the center of the colony surrounded by a less dense area of a brownish black color that became less intense as a thin, translucent margin was approached. In some instances the surface appeared slightly granular. In most cases isolated colonies of this type remained attached when normal salt solution was poured over the plate. Those that came off, did so with difficulty, leaving no pit on the agar. We think that this type corresponds closely morphologically to "the second variety of colony" described by Griffith.[37] This corresponds also to the "first variety" of colony given by Lancefield's group A hemolytic streptococci, when grown in Bacto Peptone (Difco) blood agar plates, described by Dawson, Hobby and Olmstead.[21]

2. The second type was similar to the first in many respects. The main difference was that the surface was stippled. Frequently the stippling was coarse, though often rather fine. Some of the colonies of this second type came off the medium as delicate white discs leaving tiny but macroscopically visible markings, when saline was poured over the plate. Many showed a waxy appearance. This appearance corresponds morphologically to that described by Todd[91, 94] and designated by him as the "matt" colony.

3. This type of colony was only occasionally encountered. It was large, round, soft, transparent, slightly raised and watery in appearance. Morphologically it corresponds to the "watery" colony described by Griffith,[37] the pseudo-glossy of Todd and Lancefield[93] and the mucoid of Dawson, Hobby and Olmstead.[21]

4. Five strains producing definitely rough colonies shortly after isolation from the animal tissue were encountered. Their characteristics were the following:

Strain A 23 (group A): isolated from pleural exudate which showed short Gram positive chains on direct smear. Culture of the pus on blood agar, after 36 hours at 37° C., gave colonies that looked very rough and dry macroscopically. Under low power, by artificial transmitted light, the colonies showed a central depression surrounded by a raised dark ridge that merged into a thinner watery translucent, very irregular margin. Gram positive cocci, mainly in pairs. Occasionally a short chain. Toxigenic, fibrinolytic, virulent for mice, *pyogenes* (Holman's classification).

Strain M 16 (group G): from normal throat. Rough, waxy appearance, 1 mm. in diameter, raised. Low power: a slightly

raised central portion, then a depression and finally an elevated marginal ridge. The entire colony could be easily removed with a needle from the surface of the agar. Gram positive cocci mainly in irregular clusters. No chains. Weakly fibrinolytic, virulent for mice, non-toxigenic, *anginosus* (Holman's classification).

Strain A 17 (group A): from abscess of scalp. Pus streaked on blood agar plate gave a pure culture of hemolytic streptococci. Low power: colonies definitely rough and papillated with irregular margins. There was a central depression surrounded by a very prominent dark ridge (transmitted artificial light). When saline was poured over the plate the colonies came off easily leaving a perfect cast of their under surface in the agar. Low virulence for mice, fibrinolytic, toxigenic, *pyogenes* (Holman's classification).

Strain M 24 (group A): from acute tonsillitis. Colonies small (less than 1 mm.) waxy, raised. Viewed through a hand lens they were definitely rough with a central depression surrounded by a marginal ridge. When saline was poured over the plate the colonies came off easily leaving their perfect cast. Mainly Gram positive cocci but occasionally Gram negative elements also present. Few short chains. Some balloon forms. Low virulence for mice, strongly fibrinolytic, toxigenic, *pyogenes* (Holman's classification).

Strain F 1 (group C): from ulcer in rectum. This colony was smooth upon isolation. About one and a half years after isolation 0.4 c.c. of a 20-hour broth culture was inoculated intraperitoneally into a mouse. We cannot say, however, if the colony was in the smooth phase when the inoculation was made. The animal died ten days after injection and, at autopsy, abundant cheesy exudate was found in the peritoneum between the liver and the spleen. Cultures of this exudate yielded a hemolytic streptococcus that gave the same reactions as the strain inoculated (lactose, mannitol, salicin and sorbitol negative and trehalose positive) and which belonged to group C as the strain injected. The colonies were raised, and there was a small central protuberance rising slightly above the surface of the colony which was very rough. When saline was poured over the plate the colonies came off very readily leaving an imprint in the agar closely resembling their surface. The biological properties of this rough variant were not studied except for the fermentation reactions mentioned above.

The observations on these five strains do not justify any definite conclusions, but it is interesting to note the similarity between the morphology of the three group A strains as contrasted with the

different appearance of the C and G strains. The morphology of the C and G colonies was also different.

We have been unable to discover any definite relationship between the morphology of colonies on blood agar and biological activities of the streptococci studied.

Dawson, Hobby and Olmstead[21]* in their study of "variation in the hemolytic streptococci" have attempted to discover the relationship of the different phases to some of the biological activities of the organisms. They were unable to detect any difference in the fibrinolytic activity and the hemolysis in pour plates between the R, S and M variants of Lancefield's group A hemolytic streptococci.

Appearance of growth in broth. The appearance of growth in tryptic digest beef infusion broth, in 0.05 per cent glucose neopeptone beef infusion broth and in 1 per cent glucose bacto peptone beef infusion broth was of four general types in the strains studied:

1. Homogenous with a powdery sediment.
2. Very finely granular (turbidity of medium and sediment).
3. Granular (Growth against one side of the tube with or without slight turbidity. Granular sediment).
4. Floccular (Growth against side of the tube and fluffy sediment. Settles readily when disturbed. Medium perfectly clear).

The size and consistency of the floccules and the granules varied greatly. Some went readily into a homogenous suspension when the tube was gently shaken, others necessitated vigorous shaking and in some instances the granules were impossible to disintegrate.

Morphology and staining. In direct smears from exudates hemolytic streptococci were found as single cocci, diplococci, irregular clusters of chains of varying length. In pus and in the serous exudate from ulcers the organisms were most commonly seen in chains or in the diplo-form. The organisms in direct smears from the sources mentioned above were always found to be Gram positive.

Irregular clusters were very frequently seen in the peritoneal exudate of mice that died as the result of the intraperitoneal inoculation with broth cultures of the organism. The cocci in many cases were elongated giving the appearance of a short bacillus. Gram stains frequently revealed the presence of both Gram positive and Gram

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* These authors propose a new terminology. The relationship between the new and previously accepted terminology is given by the authors in the following scheme:

(1) M (mucoid)—formerly S (smooth)
(2) S (smooth)—formerly R (rough)
(3) R (rough) —not previously described

negative cocci in the same chain. This was also frequently seen in 24 to 48 hour old cultures in the broth media mentioned above. However, when transplants were made to fresh broth and young 10 to 12 hour cultures were stained, the organisms were always Gram positive.

When streptococci from pathological material, from broth cultures, etc., were cultured on solid media the formation of chains was to a great extent inhibited giving rise in most cases to colonies which were mainly composed of irregular clusters or cocci found singly or in pairs. Chains were only occasionally seen. These colonies when transplanted to a suitable liquid medium produced chains in the great majority of cases.

In some cases the granules in the broth culture were so compact that the smears showed amorphous black masses in which the elements were so closely packed together that the individual cocci were only seen at the periphery of these large masses.

All this has been observed again and again by all investigators working with hemolytic streptococci in different parts of the world, and if we repeat it is with the sole purpose of showing that in this respect the hemolytic streptococci isolated in Puerto Rico behave in no different way.

SEROLOGICAL GROUPING

Lancefield's precipitin test is undoubtedly the most useful and practical method available at present for the classification of streptococci, and together with Griffith's[37] slide agglutination test for the identification of the different types within the hemolytic group, constitutes a most valuable tool in the study of the epidemiology of hemolytic streptococcus infections.

We have employed Lancefield's precipitin test for grouping the strains of hemolytic streptococci isolated in Puerto Rico.

The methods utilized for the preparation of the extracts, the immunization of animals and the technique of testing are those described by Lancefield.[54] The group sera used in the beginning of this work as well as the strains used for the immunization of rabbits and for testing their serum for anti "C" antibody were kindly supplied by Dr. Lancefield.

Following Dr. Lancefield's advice, based upon the findings of Bliss, subsequently published in 1938,[8] the suspensions for the preparation of hydrochloric acid extracts and those used in the immunization of rabbits were washed twice in normal saline to mini-

mize the occurrence of non-specific reactions. Cross reactions were rarely encountered except in the case of group C and G extracts with group G and C antiserum. Non-specific ring formation was frequently observed with C extracts and G antiserum and with G extracts and C antiserum. This was sometimes puzzling but this difficulty was obviated in the majority of cases by frequent examination of the tubes for the appearance of the ring. The specific reaction invariably appeared first and was always more intense. That this was the case was corroborated by repeating all the tests giving cross reactions utilizing extracts prepared by Fuller's[34] method. When formamide extracts were used the group C and G non-specific reactions were totally eliminated. Group B formamide extracts, however, gave cross reactions with groups A, C and G sera (other group sera not tested) as pointed out by Fuller, but the non-specific precipitation was not strong enough to mask the specific reaction.

When sera of good titer were used definite rings appeared promptly and further incubation was unnecessary. Likewise dilution of the extract was usually unnecessary.

The reactive substance in the HCl extracts is very stable. Extracts stored for four and a half years in the ice box in ordinary glass tubes stoppered with paraffined corks and without preservative gave as good reactions as when they were prepared.

Table III shows the group distribution among the strains studied.

A comprehensive study of Lancefield's groups among the hemolytic streptococci found in the respiratory tract of healthy persons has been undertaken by only a few investigators. Table IV summarizes the results obtained by some of these workers. Our figures for normal throats and for the throats of monkeys as well as those of Seegal, Heller and Jablanowitz[73] for rhesus monkeys are also included for comparison.

Fourteen per cent of the strains of beta hemolytic streptococci isolated from normal human throats, 22.6 per cent of strains from the throats of the general population (cases with definite symptoms of throat trouble not included), and 41.7 per cent of the strains from the throats of normal monkeys belonged to group A.

It has been very difficult for us to discern from some of the reports of other workers whether throats with such abnormalities as a hyperemic pharynx, mild chronic tonsillitis, etc., but free from actual symptoms were considered normal. It is apparent, however, that the incidence of group A strains among the beta hemolytic streptococci isolated from normal throats, and from throats in gen-

eral, in the tropical island of Puerto Rico is lower than the incidence among strains cultured from a similar source in temperate climates. Davis and Guzdar[20] found that 35.8 per cent of the hemolytic streptococci isolated from normal throats of Chinese in the subtropical city of Hong Kong were group A. This percentage occupies an intermediate position between the percentage obtained by us in Puerto Rico and that obtained by the majority of workers in temperate regions.

The researches of a number of investigators[55, 75, 71, 18, 19] have shown that the nose and throat of the patient and of contacts constitute the most probable source of infection in puerperal sepsis. The relatively low incidence of puerperal sepsis in Puerto Rico due to the streptococcus may be explained, at least in part, by a correspondingly low incidence of potentially pathogenic group A hemolytic streptococci in the upper respiratory passages of normal individuals on this Island.

It is interesting to note that out of 28 strains isolated from cases of lymphangitis of the lower extremities, clinically indistinguishable from the so called acute lymphangitis attack of filariasis, 26 were group A. Floch[30] recently studied two strains isolated from cases of lymphangitis in Guadaloupe. Both were group A but did not belong to any of Griffith's types.

It must be noted that out of five strains obtained from cases of mild sore throat only one was group A, the other four being two group G and two group C strains. Out of eleven strains from severe sore throat six were group A, four group C and one group G. Of the fourteen strains obtained from excised tonsils nine were group A and the other five group C. The incidence of group A strains isolated from these pathological sources is undoubtedly lower than it would be expected.

Kodama[51] has shown that group A, C and G strains of hemolytic streptococci can be found at the same time in the same throat. There exists, therefore, the possibility that we may have isolated the non-specific organism in some of these cases. In two instances, however, we feel sure that group C and G strains respectively were the specific organism concerned. Both were cases of severe acute tonsillitis. In both, hemolytic streptococci were present in large numbers and in practically pure culture. As the original plates had been kept in the ice box we were able to go back to them and check the grouping by testing several colonies. Six colonies were transplanted to broth from each plate and the precipitin tests repeated with the

same results as before. This showed that we were dealing with tonsillitis associated with a group C and a group G hemolytic streptococcus respectively. The group C strain (M 89) as well as the group G strain (M 45) were definitely virulent for mice. Both failed to produce an erythrogenic toxin reactive on the skin of a susceptible white goat. Both produced a potent fibrinolysin. Strains W 1 and F 1, isolated from an abdominal wound and from an intestinal ulcer respectively, also belonged to group C. We have not encountered group C strains associated with other pathological conditions outside of those referred to above.

Group G strains have been isolated in two instances from small ulcers during acute attacks of lymphangitis and also twice from fatal septicemias. It was interesting to observe that the two lymphangitis strains (L 13 and L 21) were associated with hemolytic staphylococcus in the lesion and that they were very atypical in their biological characteristics. Of the two strains obtained from the blood one was from a case of agranulocytosis and the other was from a case of puerperal sepsis.

Lancefield and Hare[55] isolated a group G hemolytic streptococcus from a fatal case of septicemia. They stated that "the patient was also suffering from an overwhelming infection with *Staphylococcus aureus*, and it is possible that the hemolytic streptococci were secondary invaders." They also noticed that "biochemically this strain was very atypical." We have observed in many instances this atypical behavior of hemolytic streptococci isolated from tissues in which they have been associated with *Staphylococcus aureus*.

Group B streptococci were encountered only twice. One strain (M 86) was cultured in moderate numbers from the throat of a man who gave a history of previous sore throat but who had no symptoms at the time the culture was taken. The other strain (S 5) was a double zone hemolytic streptococcus obtained in pure culture from the blood of a fatal case of puerperal sepsis and about which more will be said later. It must be remembered, however, that we fished from primary throat cultures only those deep colonies showing good beta hemolysis at the end of 24 hours incubation. Most group B strains produce poor zones of hemolysis and there is the possibility that group B colonies may have been overlooked.

Bovine mastitis is very common in Puerto Rico. The causative organism in the vast majority of cases was found to be *Streptococcus agalactiae* (group B). Unpasteurized milk in the market is no doubt heavily contaminated with *Streptococcus agalactiae*. This organism

had been considered innocuous for human beings, but lately evidence has been accumulating that tends to show that *Streptococcus agalactiae* may be a potential human pathogen.[19, 39, 10, 33, 12]

Groups D and E have not been encountered in the material studied. Minute beta hemolytic streptococci (group F)[7, 57, 58] were occasionally seen in throat cultures but no systematic study was made of them. The incidence of groups H and K[40] in normal throats in Puerto Rico has not been investigated.

We have obtained group H strains in pure culture from the blood of two cases of rheumatoid arthritis. Both strains were non-fibrinolytic, non-virulent for mice, did not produce either a soluble toxin or a soluble hemolysin, one produced a homogenous turbidity but the other gave a granular growth in tryptic digest broth. One was *anginosus*, the other was *equi* by Holman's classification. One fermented trehalose and not sorbitol, the other failed to ferment either trehalose or sorbitol. Deep colonies produced alpha prime hemolysis after 36 to 48 hours aerobic cultivation at 37° C. in 3 per cent rabbit blood agar.

HEMOLYSIS

In the study of no other phase of the complicated biology of streptococci have the results obtained by different workers been so conflicting and confusing as in the investigations concerning streptococcal hemolysin. These discrepancies in results have arisen mainly from the fact that different investigators have employed different techniques and factors of importance have been ignored, such as composition of the medium, age of the culture, kind and amount of blood, the inherent ability of different strains to produce varying amounts of hemolysin and the existence of essentially different hemolytic principles.

We shall not attempt to review the literature on the subject. This has been done in great detail by the Thomsons[85] up to the year 1927. Information about the more recent developments will be found in the publications of Todd,[95, 97, 98] Hodge and Swift,[43] Weld,[105] Brown[11, 12] and Smith.[77, 78]

Plate hemolysis. The same blood agar medium used for primary isolations from the throat was also utilized for this test. Transplants were made from stock cultures to 5 c.c. of tryptic digest broth and incubated aerobically for 18 hours at 37° C. From this growth a poured rabbit blood agar plate was made with a very small inoculum so as to obtain well isolated deep and surface colonies. The plate was

incubated aerobically at 37° C. for 24 hours when it was examined for the presence of hemolytic zones around deep and surface colonies.

In practically all cases (of beta hemolytic strains) well isolated surface colonies gave rather poor hemolysis while deep colonies were surrounded by wide zones of true beta hemolysis.

One hundred and sixty strains from different human sources and from the throats of normal monkeys producing beta hemolysis upon isolation were studied. Eighty-five strains were group A, 48 were group C, 26 were group G and one was group B. All these strains have been kept continuously on 3 per cent rabbit blood agar slants for periods ranging from six months to six years. Transplants have been made at approximately three months intervals. So far we have not observed in a single case any change in the ability of these strains to produce beta hemolysis. We took sixty of these strains to the Hygienic Laboratory of the University of Michigan and kept them there for 13 months during which they were transplanted five times on blood agar slants in ordinary glass test tubes. When they were brought back to Puerto Rico and retested for the production of plate hemolysis this was found in every instance to have remained unaffected. The permanency of this property in the strains studied has been striking. We want to emphasize the fact that we were dealing with strains that produced typical beta hemolysis upon isolation from the animal tissues and that although we have been working with mass cultures we always plated out the original cultures several times, as has been pointed out before, in an attempt to minimize the possibility of mixed cultures.

It was observed that in most cases the hemolyzed areas around group C and group G colonies were larger (3–4 mm. in diameter) than those around group A colonies (2–3 mm. in diameter). We notice, however, that a similar observation has already been made by Seegal et al.[73] in 1936 and by Kodama[51] in 1937.

Six strains that did not produce typical beta hemolysis in primary cultures have been included in this study. Of these, three were group A (L 14, M 74, D.M. 1), two were group G (L 13 and L 21) and one was group B (S 5).

Strains L 13, L 14 and L 21 were associated in the lesion with hemolytic *Staphylococcus aureus*. They were poorly hemolytic upon isolation but upon cultivation on blood agar their hemolytic power gradually increased until they showed typical beta hemolysis. Strain M 74 behaved in the same way.

In Strain S 5, when isolated in 1936, the appearance of the colonies

on rabbit blood agar, after 24 hours incubation at 37° C. and overnight refrigeration at 10° C., was typical of the double zone beta hemolytic streptococci first observed in 1913–1914 by Smith and Brown[79] in strains from cow's milk and by Jones[46] in strains from the throat of horses in 1919, and later described by Brown in 1934[10] and in 1937.[11] Surface colonies on blood agar (24 hours) were relatively large (1.5 mm.-2 mm. in diameter), rather flat, smooth, soft, button-like, with slight depression in the center and a small dark central spot. Gram stain showed short chains and small clusters of Gram positive cocci. Sodium hippurate was hydrolyzed, methylene blue in milk was not reduced, it gave a final pH of 4.4 in 1 per cent glucose broth, it was non fibrinolytic when tested on a susceptible human plasma clot, it did not produce a soluble erythrogenic toxin reactive on the skin of a white goat, it gave a negative tube hemolysis with the whole culture, the untreated supernatant and the reduced supernatant when grown for 18 hours in tryptic digest broth; 0.4 c.c. of an 18 hour broth culture killed mice in one day, it fermented salicin and trehalose but did not ferment lactose, mannitol or sorbitol. This strain is therefore similar in many respects to group B strains found in cow's milk and known to be associated with bovine mastitis.[10, 11, 56] After three years the colonies have apparently lost their ability to produce the double zone appearance in rabbit blood agar. At present they look just like ordinary *Streptococcus agalactiae.* Brown,[11] however, emphasizes the permanency of the double zone appearance. He gives photographs of colonies of the same strain taken in 1915 and again in 1936 to illustrate the constancy of the phenomenon.

The pathogenic significance and serological grouping of double zone beta hemolytic streptococci has been discussed by Brown[12] in a recent publication.

During a study of the streptococci associated with bovine mastitis in Puerto Rico[63] a peculiar type of colony has been encountered. In its serological grouping and some other biochemical and cultural properties it resembles double zone beta hemolytic streptococci. However, in two respects it is quite different: (a) in this type of colony the second hemolyzed zone is replaced by a bluish black ring 1–2 mm. in diameter, and (b) the formation of this ring does not necessitate refrigeration. It appears after 24 hours at 37° C. around both deep and surface colonies, and remains unaltered after overnight storage in the ice box. Two years after isolation the appearance of colonies on rabbit blood agar has remained the same.

Strain D.M. 1 was obtained in pure culture at autopsy from the heart blood of a rhesus monkey that had been dead for about one hour. This organism was incriminated by the pathologist as the cause of death. Upon isolation its macroscopic appearance in a poured blood agar plate was that of an ordinary green producing streptococcus. Only very slight hemolysis was exhibited by deep colonies and the cells inside this partially hemolyzed zone showed definite greenish discoloration. However, tube hemolysis tests on the 18 hour growth in streptolysin broth gave the following results: whole culture, complete hemolysis in fifteen minutes; untreated supernatant, delayed (one hour) but complete hemolysis due perhaps to the presence of some streptococci in the supernatant after centrifugation; reduced supernatant, very prompt (less than one minute) complete hemolysis.

This strain was tested again ten days after isolation and there was a definite hemolysis around deep colonies after 24 hours incubation. The organism was kept on a 3 per cent rabbit blood agar slant on which it gave good growth but when transplants were attempted 40 days later the organism did not grow and the strain was lost.

Howell[45] and Todd[92] have effected a hemolytic to viridans change by mouse passage. This change, however, was temporary. The green variants reverted to the hemolytic forms upon artificial cultivation. Todd stated that "the passage culture, though hemolytic in the absence of oxygen, establishes some unknown condition in the presence of oxygen which abolishes hemolysin and, when grown symbiotically with an ordinary strain of hemolytic streptococcus which is capable of forming hemolysin in the presence of oxygen, it reduces the hemolytic titer of the symbiotic culture either by destroying hemolysin or by preventing its formation."

Fuller and Maxted[35] have recently investigated "the production of hemolysin and peroxide by hemolytic streptococci in relation to the non-hemolytic variants of group A." It is apparent that the green variants as well as most of the hemolytic forms produce hydrogen peroxide during their growth in serum broth cultures, but in the former hydrogen peroxide appears earlier than the hemolysin, thus inhibiting hemolysis, while in the latter, hemolysis appears first.

We have in many instances failed to isolate true beta hemolytic streptococci from throats in which infection with this organism was clinically suspected. In many cases the primary plates showed large numbers of slightly hemolytic streptococcus colonies that exhibited varying degrees of greenish discoloration. There exists the possibility

that these colonies might have been green variants of group A beta hemolytic streptococci. In this connection Fuller and Maxted[35] state: "The practical point which emerges is that these green variants are hemolytic streptococci of undiminished virulence, and cultural conditions should be chosen so that they produce beta hemolytic zones on a blood agar plate." These authors recommend the employment of anerobic cultivation to obviate this difficulty. Anerobic culture methods are also strongly advocated by Fry,[33] Hare[41] and others.

Strain D.M. 1 was virulent for mice, it produced an erythrogenic toxin reactive on the skin of a white goat, belonged to Lancefield's group A and it dissolved completely susceptible human plasma clot in 40 min., but caused only very slight dissolution of rhesus monkey plasma clot in 24 hours, a fact which is strongly suggestive of the ultimate human origin of the strain.

Many claims have been made of the conversion of hemolytic streptococci to the viridans and anhemolytic forms and vice versa. We will not enter into discussion of this point here. We will limit ourselves to saying that during the course of this work such permanent changes have not been encountered.

Tube hemolysis. At the beginning we used tryptic digest broth[24]* to grow the streptococci for this test. Later, for definite reasons, streptolysin broth was also utilized. First we will give the results obtained with tryptic digest broth cultures and then we will discuss the results obtained with streptolysin broth cultures.

An 18 hour growth in 5 c.c. of tryptic digest broth was employed. Rabbit blood was collected in 1 per cent sodium citrate in 0.9 per cent saline. The washed cells were suspended in enough 0.9 per cent saline to give a concentration of approximately 2 per cent.

The whole culture (W), the clear unreduced supernatant (S u) and the reduced supernatant (S r) were tested in each case as follows: 1 c.c. of the whole culture was placed in a Wassermann tube. The rest of the broth culture was centrifuged until a clear supernatant was obtained.** One c.c. of this supernatant was placed in another Wassermann tube and the rest was transferred to a clean test tube and a pinch of sodium hydrosulphite was added. The tube containing the hydrosulphite was then placed in a Novy jar or in a

* No indicator added.

** The time and speed of centrifugation varied according to the nature of the growth. Granular or floccular growths centrifuged most readily and strains giving homogenous or finely granular growths offered the greatest difficulty.

vacuum desiccator and maintained under a partial vacuum (at room temperature) by means of a suction water pump for about ten minutes, or until no more bubbles came off the liquid. One c.c. of this reduced supernatant was placed in a third Wassermann tube. To each tube 0.5 c.c. of the red cell suspension was added, well mixed and the tubes were placed in a water bath at 37° C. They were examined at short intervals. The time required for complete hemolysis was recorded and when necessary the tubes were left in the water bath for one hour at the end of which those showing incomplete hemolysis were centrifuged and readings were made. No attempt was made at a quantitative determination of the hemolysins. A rough idea was obtained from the rapidity with which the hemolysis was effected.

The results were recorded as +, ++, +++, or ++++ according to whether approximately ¼, ½, ¾ or all the red cells were lysed, as estimated from the bulk of sedimented red blood corpuscles after centrifugation. Traces of hemolysis were recorded as ± and — to signify absence of hemolysis.

Table V shows the results obtained with representative strains of groups A, C and G tested shortly after isolation.

Table V contains several points which are worthy of further consideration: None of the group A strains gave a hemolytic supernatant after reduction with sodium hydrosulphite. As a control the well-known Dochez scarlet fever strain N.Y. 5, which belongs to Lancefield's group A, was used in these tests and gave consistently complete hemolysis both with the whole culture and the reduced supernatant; the untreated supernatant was not hemolytic. We have not tested, *in this same medium*, streptococci isolated in temperate climates and, consequently, the results are presented without further discussion. In all those cases where a positive hemolysis is given with the whole culture but which are negative with the reduced and untreated supernatant, the presence of an endocellular hemolytic principle is postulated. In this connection the work of Smith[77] should be mentioned which strongly supports his conclusion "that hemolysis by living streptococci during a one-hour period at 37° C. is not due to the continued multiplication of the cocci."

In some cases the untreated supernatant produced a hemolytic effect of varying intensity which disappeared upon reduction with sodium hydrosulphite. Some of the cultures that behaved in this way were grown in 200 c.c. of medium. They were filtered through Berkefeld candles "W" (the first 100 c.c. to pass the filter were dis-

carded). When tested the unreduced filtrate produced no hemolysis. This led to the conclusion that the hemolytic effect of the unreduced supernatant, in most instances at least, was due to streptococci remaining in suspension even after prolonged centrifugation. In these cases, as has been pointed out before, hemolysis is inhibited by the addition of sodium hydrosulphite. Smith[78] in discussing the hemolytic properties of his neopeptone broth culture stated: "The medium here developed gives an extraordinarily high yield of streptolysin but it remains quantitatively associated with the organisms. Despite the fact that no lysin can be detected in filtrates or centrifugates it is liberated almost entirely by shaking the washed organisms in serum for 3 minutes." ". . . there is no evidence of the formation in this medium of filtrable hemolysin. Attempted reduction of filtrate or whole culture with sodium hydrosulphite always results in rapid destruction of the hemolysin."

In view of the results with tryptic digest broth, as shown in table V, and of observations with the *phosphate-glucose-bicarbonate-proteose* broth of Swift and Hodge,[83] as shown in table VI, it is believed Smith did not obtain hemolytic filtrates after reduction because he was using a strain which was not able to elaborate the hemolytic substance in his medium; this does not mean, however, that all strains will behave in the same manner.

No apparent relationship was observed between the hemolytic properties of the different strains and their source; also no relationships could be detected either between hemolysis and mouse virulence or between hemolysis and any of the biological characteristics studied.

In marked contrast with the group A strains the majority of the group C and G streptococci produced a supernatant which was hemolytic after reduction. There is the possibility of this being quantitative in character, but it is not likely to be so because all three groups grew equally well in this medium. Even if it were a purely quantitative relationship there is undoubtedly a great variation between different strains in their ability to elaborate the different hemolytic substances.

Smith[77] while discussing the optimum peptone concentration for the production of hemolysin in his neopeptone broth stated: ". . . it is pertinent to point out that exhaustion of the neopeptone herein described by growth in it of a group C hemolytic streptococcus leaves the medium still able to produce hemolysin by a group A streptococcus. Group A organisms will exhaust the medium for both

group A and group C streptococci, and all group A organisms are apparently identical in this respect. This argues in favor of separate lysin precursors for at least two groups of hemolytic streptococci."

Attention should be called to the fact that so far in this work the results refer only to lysin production in tryptic digest broth. No peptone or glucose was added to this broth although a small amount of both may have been present, the medium being a mixture of beef infusion and a tryptic digest of casein.

The streptolysin broth of Swift and Hodge[83] is a modification of the formula of Todd and Hewitt[96] and consists essentially of a beef heart proteose peptone infusion to which a phosphate-glucose-bicarbonate mixture, sterilized by filtration, is added.

A large number of strains representative of groups A, C, and G were grown in this medium and the hemolytic properties of the cultures investigated exactly as already described. The results are shown in table VI.

Seventy-two group A strains were tested when grown in streptolysin broth. In this medium thirty-six (50 per cent) gave a hemolytic reduced supernatant and four (6 per cent) a doubtful reaction. As a comparison, twenty-two strains of group A hemolytic streptococci isolated from different pathological conditions in temperate climates were also tested.* Ten (45 per cent) gave a hemolytic reduced supernatant; seven (30 per cent) were negative and five (25 per cent) gave a doubtful reaction.

It is interesting to note that some group C and G strains did not produce a supernatant hemolytic after reduction when grown in tryptic digest broth but did give a hemolytic supernatant after reduction when grown in streptolysin broth. Occasionally strains were encountered which did not give a hemolytic supernatant in streptolysin broth after addition of sodium hydrosulphite but these also failed to do so in tryptic digest broth.

From these results the following conclusions seem justified: Many group A and most group C and G strains, when grown in the streptolysin broth of Swift and Hodge,[83] elaborate two different hemolytic principles (a) one which remains attached to the bacterial cell and whose activity is in some way inhibited by the addition of sodium hydrosulphite and (b) another which is found in the medium and deteriorates rapidly but whose hemolytic activity is restored by

* Obtained through the kindness of Dr. Alice Evans of the National Institute of Health in Washington, and Mr. R. B. Lindbergh of the Hospital for Contagious Diseases at Ann Arbor.

reduction with sodium hydrosulphite. These two substances can be elaborated at the same time by the same strain. Todd[97] has shown by serological methods that group A strains produce two different hemolysins. Todd's claims appear to be substantiated by our results. Whether or not all group A strains are potentially able to elaborate these two substances under proper conditions, is, of course, a different matter.

Occasionally strains have been encountered which give a markedly hemolytic supernatant and this property is retained after the addition of sodium hydrosulphite, suggesting the presence of an additional oxygen stable hemolytic principle. However, we have not made quantitative determinations of the hemolysins present in the treated and untreated preparations and there remains the possibility of the presence of active streptolysin in the reduced filtrate which may lead one to wrong conclusions.

The stimulating effect on hemolysin production of proper concentrations of peptone has been recognized for a long time. The importance of this substance and of glucose in the production of hemolysin by streptococci has been recently emphasized by Smith.[77, 78]

It is apparent that the hemolysins produced by streptococci of tropical origin are similar to those obtained in temperate regions.

FERMENTATION REACTIONS

We have used lactose, mannitol, salicin, trehalose, and sorbitol as test substances. Tryptic digest beef infusion broth,[24*] in which all the strains studied grew well, has been used as the basic medium. Ten or twenty per cent solutions of the test substances were made in distilled water and sterilized by filtration. Enough of these sterile solutions was added to about five c.c. of the basic medium in fermentation tubes to give a final concentration of approximately 1 per cent. The tubes were incubated for 48 hours at 37° C. to check sterility and they were inoculated with 0.05 c.c. of an 18 hour old growth in plain tryptic digest broth of the strain to be tested. A tube of plain tryptic digest broth was inoculated at the same time and used as a control. The tubes were incubated aerobically at 37° C. for forty-eight hours at the end of which two drops of Andrade's indicator were added to each and readings were made.

A total of 175 strains was examined. One hundred and fourteen (65 per cent) were *Str. pyogenes* of which 90 were group A, 22 group

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* Indicator omitted from the basic medium.

C, and 2 group B. None of the 34 group G strains were *Str. pyogenes*. The majority of the *pyogenes* strains were associated with pathological conditions. Some were found in normal throats and in the throats of normal rhesus monkeys. All the 24 strains from abscesses and other suppurating lesions and 24 out of the 28 lymphangitis strains fell in this group by their fermentation reactions.

Forty strains (23 per cent) belonged to *Str. equi*. Of these, 14 were group C and 26 were group G. Of the remaining 21 strains, 17 were *Str. subacidus* (9 group C and 8 group G); 3 were *Str. anginosus* (2 group A and 1 group G) and one was *Str. infrequens* (group A).

Only one of the group G strains fermented lactose. This is in good agreement with the results of Davis and Guzdar[20] who found that most of the G strains cultured from the throats of Hong Kong Chinese were lactose negative. Similar results were also obtained by Seegal et al.[73] with strains from the throats of normal rhesus monkeys: out of five group G strains, four were *equi*. On the other hand our results are in complete disagreement with those of Hare[40] and of Lancefield and Hare.[55] Hare found that out of 13 group G strains, isolated from normal throats in England, 11 were *pyogenes*. Many of our C strains (14 out of 45) were *Str. equi* and 9 were *Str. subacidus*. Again our results agree with those of Guzdar and Davis who found that "a considerable number" of their group C cultures were non-lactose fermenters; with those of Plummer,[68] who reported that of eight group C strains of human origin six did not ferment lactose; and with those of Evans[28] who found that among the human trehalose fermenting group C strains studied by her many failed to ferment lactose. Hare,[40] however, found that all the group C strains (fifteen) of his collection of 100 strains from normal throats were *Str. pyogenes* by Holman's classification.

The reactions of our strains have not only been clear-cut but they have shown a remarkable constancy. Many of them have been tested at from 3 to 15 months' intervals during the last four or five years always with the same results.

For the discrepancy between our results and those of the investigators cited we have no explanation to offer. However, there is one point that we want to emphasize here in this connection: the difference in the composition of the basic medium employed for the fermentation reactions. Seegal et al. used plain broth; Davis and Guzdar employed a modified Hiss's serum water medium (25 per cent serum peptone water); Hare does not give the medium used in the paper referred to above, but in a previous work of this same

nature[39] he used Hiss's serum water; Evans and Plummer both utilized a sugar-free infusion broth, and Lancefield and Hare used Hiss's serum water.

The quality of the medium rather than merely the presence of abundant growth appears to be the important factor concerned.

When we speak of *Str. pyogenes* and *Str. equi* in the above discussion we are limiting the meaning of these terms to hemolytic streptococci that ferment lactose and salicin but fail to ferment mannite in the former, and to organisms that ferment salicin but do not ferment either lactose or mannite in the latter.* When these limitations are not indicated these terms may have quite different meaning. This has been and still is the source of great confusion in the literature. *Str. equi* in the more restricted sense, and the generally accepted correct one, refers to that group of hemolytic streptococci which causes strangles in horses, ferments salicin but does not ferment lactose, mannitol, trehalose, and sorbitol. It is in this sense that the term is employed by Ogura,[64] Ewards,[24] Sherman[76] and Evans.[26]

All the strains tested fermented trehalose and not sorbitol except three. This is in accordance with the results of Evans,[28] Edwards[24] and Plummer.[67]

The three strains that failed to ferment trehalose were group C. One (D 1) was from a case of infectious eczematoid dermatitis, it also failed to ferment sorbitol, lactose, mannitol and salicin. Another (C. Mo. 72), from the throat of a normal rhesus monkey, was *Str. pyogenes* according to Holman's classification. Sorbitol was fermented. The other (M 89), from a severe tonsillitis, was *Str. pyogenes* (Holman) and fermented sorbitol. It was highly virulent for mice.

Among "409 strains of groups A and C isolated from human sources," Evans[28] encountered only one that fermented sorbitol and failed to ferment trehalose and of which she said the following: "The single human strain which ferments sorbitol but not trehalose was received from Hungary with a history of having been isolated from a scarlet fever throat. Since this strain is so unusual as a human strain but agrees with animal strains, it seems probable that sometime there may have been some error in regard to it." Evans did not state whether this strain was group A or group C.

Aside from these two we do not know of any other trehalose negative, sorbitol positive beta hemolytic streptococcus found to be associated with definite human pathological conditions.

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* Holman's classification 44.

Grace and Grace,[36] in their study of the fermentation reactions (Holman) of 68 strains of hemolytic streptococci isolated in the tropics (British Guiana), mainly from abscesses and the blood stream, found that 61 were *pyogenes*, 5 were *subacidus*, 1 was *anginosus*, and 1 *equi*. These findings are in close agreement with ours with strains from similar sources. Our results also agree in a general way with those of some of the investigators[44, 5, 6, 29] who have worked with streptococci isolated in temperate climates. However, a comparison of our findings with those of the majority of other workers has been rendered impossible due to the great variation in the materials and methods employed and the difference in the source of the strains studied.

In spite of the difficulties encountered in comparing the results of the present study with those of others, it seems justifiable to say that the fermentative reactions of hemolytic streptococci isolated in Puerto Rico are not materially different from those of hemolytic streptococci isolated from similar sources in temperate regions.

REDUCTION OF METHYLENE BLUE. HYDROLYSIS OF SODIUM HIPPURATE. FINAL PH IN GLUCOSE BROTH

These tests, in conjunction with the fermentation of trehalose and sorbitol, have been used by many workers as an aid in the differentiation of hemolytic streptococci of human origin from strains most commonly encountered in cows, horses, and other animals. The fermentation of trehalose and sorbitol has already been discussed. The results obtained with the other tests will now be presented.

1. Methods

Reduction of methylene blue in milk. The medium for this test was prepared according to the original method of Avery.[3] A 1:500 solution of the dye in distilled water was sterilized by autoclaving at 120° C. for 15 minutes. One c.c. of this solution was added to each tube of sterile skimmed milk to obtain the recommended 1:5000 final dilution. After the addition of the methylene blue the medium was allowed to stand at room temperature for six days before inoculations were made. The tubes were inoculated with 0.1 c.c. of an 18 hour growth in tryptic digest medium of the strain to be tested and incubated for 7 days at 37° C. The tubes were examined twice daily (at 8 A.M. and 4 P.M.) for the decolorization of the dye. Shaking of the contents of the tube was avoided. In every instance in which

decolorization took place a Gram stain was made to check the purity of the culture. Decolorization usually began at the bottom of the tube and in some cases, at the bottom and side also. Results were recorded as +, ++, +++, and ++++ according to the bulk of the medium decolorized and the intensity of the decolorization. Very slight reactions were recorded as ±, and complete absence of reduction as —.

With some strains reduction was prompt, with others it was delayed, decolorization appearing only after two or more days' incubation.

Hydrolysis of sodium hippurate. Two media were used at the same time for this test 1—that described by Ayers and Rupp,[4] and 2—the one recently recommended by Coffey and Foley.[16] Five c.c. of medium were inoculated with 0.05 c.c. of an 18-hour culture in glucose neopeptone broth of the strain to be tested and incubated for 48 hours. The cultures were then tested for hydrolysis following in each case the directions given by the authors cited. Although good results were obtained with both media* the asparagine medium of Coffey and Foley gave, in general, more clear-cut results.

Final pH in glucose broth. Five c.c. of glucose (one per cent) Bacto peptone beef infusion broth** were inoculated with 0.1 c.c. of an 18-hour growth in tryptic digest broth of the streptococcus to be tested and incubated for 48 hours at 37° C. The cultures were centrifuged and the pH of the supernatant was determined with the glass electrode.

2. Results

The results obtained with these three tests are summarized in table VII.

The final pH in glucose broth varied among the individual strains of a single serological group. However, when the average values were considered, groups A and C gave higher values (pH 5.2) than the group B strains (pH 4.7) and group G strains produced a degree of acidity intermediate between the two (pH 5.0). The overlapping of the final pH among strains of different groups is too great and

* Coffey and Foley obtained variable results with the medium of Ayers and Rupp when different lots of peptone were used. We utilized peptone from a single lot.

** The glucose was dissolved in a portion of the plain medium and sterilized by filtration. Enough of this solution was added to the plain infusion broth to make a final concentration of one per cent glucose and a final volume of 5 c.c.

this test does not have differential value when individual strains are studied. We have not considered group C strains of animal origin but Edwards[24] tested a number of these strains* and came to a similar conclusion when he said ". . . the animal strains examined in this work produced a higher acidity in glucose broth than did the human strains. However, this method may not be of practical value since some of the human strains produce almost as great a degree of acidity as do the animal strains."

Table VIII shows the final pH values in one per cent glucose broth obtained by different investigators.

Evans,[26] in characterizing the type strain of *Str. equi* (group C hemolytic streptococci from strangles in horses) records as 5.2 its final pH in one per cent glucose broth.

As seen by the results obtained by the majority of the workers cited this test seems to afford no clear distinction between streptococci of groups A, C and G. Group B strains, when considered as a whole, give distinctly higher final acidity.

All the strains from human sources except two failed to hydrolyze sodium hippurate. The two exceptions were group B strains (S 5 and M 86) which also failed to reduce methylene blue and produced a final pH of 4.4 in glucose broth; all of which identifies them with the streptococci commonly obtained from the udder in cases of bovine mastitis. All the 18 strains isolated from cow mastitis hydrolyzed sodium hippurate.

Klimmer and Haupt[48] and Klimmer, Haupt and Roots[49] have reported that the low acid producing, true beta hemolytic streptococci of equine origin as well as the high acid producing streptococci of bovine origin do hydrolyze sodium hippurate when a special technique is utilized for the detection of benzoic acid in the cultures. This awaits confirmation.

Only three (4.3 per cent) of the seventy group A strains reduced methylene blue in milk. All the 20 group B strains failed to decolorize the dye. Thirty-three (75 per cent) of the 44 group C strains reduced methylene blue. Nine strains left the dye unaffected and two produced doubtful reactions. Eighteen (78 per cent) of the 23 group G strains reduced methylene blue. Our results concerning the hydrolysis of sodium hippurate and the reduction of methylene blue in milk are in close agreement, as the case may be, with those reported by investigators working with strains isolated in temperate

* Strains from strangles in horses which belong to Lancefield's group C.

regions[60, 39, 25, 84, 54] and with those of Davis and Guzdar[20] with strains isolated from normal throats in Hong Kong. On the other hand, they are in conflict with the statement made by Topley and Wilson[100] that group G strains do not reduce methylene blue. The reduction of methylene blue and hydrolysis of sodium hippurate constitute a helpful guide in the serological grouping of hemolytic streptococci by Lancefield's method.

THE PRODUCTION OF ERYTHROGENIC TOXIN

Dick and Dick,[22] in 1924, by the application of the Schick intradermal technique, were able to demonstrate the presence in the culture filtrates of streptococci from scarlet fever, of a substance reactive in the skin of susceptible persons. This toxic principle was subsequently shown to react also in the skin of some of the lower animals, especially white goats[47] and rabbits.[101]

The presence and distribution of this erythrogenic toxin among the culture filtrates of 154 strains of hemolytic streptococci isolated in Puerto Rico, as indicated by the reaction elicited by intradermal injection to a susceptible white goat, will be reported here.

In the preparation of the toxin 250 c.c. flasks containing 100 c.c. of streptococcus toxin broth[103] were inoculated with 0.5 c.c. of an 18 hour growth in glucose neopeptone beef infusion broth and incubated for 48 hours at 37° C. The cultures were centrifuged and the supernatant was passed through a Berkefeld candle "W." Ten to fifteen c.c. of filtrate were transferred to sterile test tubes and stored in the ice box until ready for use. Tests were never done with filtrates older than five months, although the toxin is quite stable at ice box temperature.

Not all goats were susceptible. Of four white goats tested two did not react, one reacted weakly, and one gave very good reactions.

The same day that tests were to be made both sides of the goat were carefully shaved with a barber's razor, the excess of soap was thoroughly washed with water and the shaved area dried with a towel. A 1:100 dilution of each of the filtrates to be tested was made with 0.9 per cent salt solution and 0.1 c.c. of this dilution was injected intracutaneously.*

Injections were well spaced in order to obviate difficulties in

* The same amounts of heated (2 hours in Arnold at 100° C.) and unheated filtrate, prepared from Dochez' N. Y. 5 scarlet fever strain were always used as controls. Some of our strains gave areas of erythema and a degree of edema equal to, and occasionally even larger than those produced by the N. Y. 5 strain; on the average, however, reactions were less intense.

reading the reactions. One c.c. tuberculin syringes and 26 gauge ½ inch needles were used. Readings were made twenty-four hours after injection. The erythema gradually faded out and after 48–72 hours it had completely disappeared. With stronger reactions a brownish pigmentation of the skin remained for some time but also eventually disappeared.

The size and intensity of the areas of erythema varied with individual strains. All filtrates giving an area less than five mm. in diameter were considered negative and all areas of only slight erythema without swelling from five to eight mm. in diameter were considered doubtful (±) reactions. Areas of definite erythema 10 mm. or more in diameter with or without swelling were considered positive. In the majority of cases erythema was accompanied by edema. The appearance of the reactions in the skin of the goat were in all respects comparable to those obtained in susceptible human beings.

We have used the same animal for all our tests with rest intervals of about three months between the different series of injections. The animal reacts at present as well as five years ago when the first tests were made.

The observations are presented in table IX.

Out of 75 group A strains tested, 57 (76 per cent) were positive, 6 (8 per cent) were doubtful and 12 (16 per cent) were negative. The production of toxin among hemolytic streptococci of group A does not seem to bear any definite relation to the source of the strain (i.e., abscesses, normal throat, pathological throat, etc.). However, it is interesting to note that all group A strains from lymphangitis produced an erythrogenic toxin except one which gave a doubtful reaction. These findings agree strikingly with those of Coffey[17] in the United States, and of Kodama[50] in Japan. These authors found that 74.3 per cent and 73 per cent respectively of the group A strains studied by them produced the erythrogenic toxin.

Davis and Guzdar[20] working with strains isolated from the normal throat of Hong Kong Chinese found that "all group A strains gave rise to areas of erythema measuring more than five mm., and of the 28 strains examined 27 gave areas more than 10 mm." (Susceptible human beings were used for the test).

Teiger and Seegal[84] found the majority, and about the same proportion, of the hemolytic streptococci isolated by them from throats in New Orleans and in New York to produce a soluble toxin active in the skin of rabbits.

Of 19 group A strains isolated by Seegal et al.[73] from the throat of normal rhesus monkeys in New York, 6 were tested for "a toxin reactive in the skin of silver fox rabbits," and all gave positive reactions. Of 8 group A strains isolated from the throats of normal rhesus monkeys during this investigation only 3 gave a good reaction when tested in the skin of a white goat; two reacted weakly and three were negative.

None of the group C strains, irrespective of source (normal human or monkey* throats, pathological throats, etc.) produced an erythrogenic toxin reactive on the skin of the goat. This was also true of all group G strains except one. The exception was a strain obtained from a person with a previous history of mild sore throat. The two are in close agreement with those of Davis and Guzdar[20] and Kodama.[50]

We think it is pertinent to quote the results obtained by Coffey** with strains isolated in Puerto Rico from recurrent tropical lymphangitis.*** The serological analysis of these strains was summarized by her as shown in table X.

It is interesting to note, as Coffey comments, "that among a relatively small number of strains, representatives of five different toxin groups should be found . . ."

In the light of these results the difficulties to be encountered in attempts at active immunization with these filtrates are obvious. A highly polyvalent filtrate must be employed.

It is also of interest to observe that antiserum prepared against scarlet fever strain No. 165 completely neutralizes the toxins produced by the majority of the lymphangitis strains tested. If antisera prepared with these strains will completely neutralize the toxin produced by strain No. 165 we do not know.

The fact that scarlet fever as it occurs in temperate climates is rare in Puerto Rico is very puzzling. Even much more so is the striking parallelism between the results obtained with the Dick test on this Island[72] and the results obtained in temperate regions where the disease is common. No matter whether we consider the Dick test as a toxin-antitoxin reaction or allergic in nature, this parallelism in the results of the Dick test is paradoxical when we

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* It is apparent, as will be seen later, that the strains of hemolytic streptococci cultured from the throats of rhesus monkeys are of ultimate human origin.

** Personal communication of Dr. A. Wadsworth to Dr. P. Morales-Otero.

*** These fifteen cultures studied by Coffey are among the 22 lymphangitis strains studied in the present work.

consider the lower incidence of hemolytic streptococci in the throats of normal persons in the tropics as compared with that obtaining in temperate climates. The non-specificity of the Dick reaction has been suggested. This is a very important and difficult problem that awaits solution.

The ability of hemolytic streptococci isolated in Puerto Rico to produce an erythrogenic toxin does not seem to differ from that possessed by strains isolated from similar sources in temperate regions.

FIBRINOLYSIS

In 1933 Tillett and Garner[86] announced the discovery of a substance in broth cultures and filtrates of beta hemolytic streptococci of human origin, that was able "to liquefy rapidly the clotted fibrin of normal human plasma." They called this lytic principle fibrinolysin. In 1934 Tillett, Edwards, and Garner[87] demonstrated the presence, in the blood of patients who had recently recovered from hemolytic streptococcus infections, of a substance which rendered the plasma clot from these patients refractory to the action of the fibrinolytic principle. This substance has been designated as antifibrinolysin.

During the past six years about one hundred papers have appeared concerning this subject. These have been analyzed by Tillett[89] in an exhaustive review. The reader is referred to Doctor Tillett's paper if details concerning this interesting subject are desired.

In the study of fibrinolysis the method originally described by Tillett and Garner[86] was followed. The streptococci were grown for 18 hours at 37° C. in tubes containing 5 c.c. of medium. One c.c. of a 1:4 dilution in 0.9 per cent saline of a susceptible plasma was placed into a Wassermann tube and one half c.c. of culture and 0.25 c.c. of a 0.25 per cent solution of calcium chloride in 0.9 per cent salt solution were added. After mixing, the tube was placed in a water bath at 37° C. (clot formation took place in from 2 to 8 minutes) and the time necessary for dissolution was noted. Plasma from the same individual was utilized throughout this work so as to obtain as constant and comparable results as possible. One or the other (or both) of two strains* known to produce a potent lysin, and uninoculated broth were carried as controls every time a determination or a series of determinations was made.

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* T 8—a group C strain obtained from an excised tonsil.
Co.—a group A strain kindly supplied by Dr. W. S. Tillett.

A total of 177* strains (88 group A, 49 group C, 31 group G and 2 group B) from human sources and 18 group B (*Str. agalactiae*) strains of bovine origin were examined.

Three different media were employed, at one time or other during this investigation, to grow the streptococci. First we used tryptic digest broth, later we changed to glucose (0.02 per cent) neopeptone (10 per cent) beef infusion broth, and finally, following Doctor Tillett's recommendation, we used glucose neopeptone beef infusion broth to which one or two drops of sterile defibrinated rabbit blood had been added previous to inoculation.

Although fibrinolysis could be demonstrated with all three media, there was a marked difference in the fibrinolytic power of the cultures in the different media. This is illustrated in table XI.

The strains that caused no dissolution or only partial dissolution in 24 hours, as well as those that were tested for the first time long after isolation, have not been included in the preceding table.

When two actively fibrinolytic strains (T 8 and Co.) were grown at the same time in these three media and tested with the same plasma, the difference in the fibrinolytic power of the cultures paralleled that observed in table XI.

Glucose neopeptone broth gave a better growth than tryptic digest broth but the most abundant growth was obtained in the blood-containing medium. We are not able, however, to say if the increase in the fibrinolytic power of the culture was due merely to the abundance of growth or if other factors were concerned. In this connection Tillett[89] comments as follows: ". . . the author has noted that when selected strains were cultivated simultaneously in samples of culture media containing different ingredients, the lytic potency of the individual strains varied, even though the amount of growth seemed to be comparable in the different kinds of media. When one considers by analogy the effect of culture media on the production by other bacteria of products such as toxins, it seems likely that the specific stimulation or impairment of the elaboration of fibrinolysin may be subject to conditions of the same order."

It is apparent that the failure reported by some investigators to obtain actively fibrinolytic cultures has been due, at least in part, to the use of unfavorable culture media. The age of the culture actually employed in the test, as well as that of the culture from which the transplant is made, is also a very important factor in this connection.

* Seven strains were not grouped by Lancefield's method.

Of the 173 strains of hemolytic streptococci tested, 164 (95 per cent) were definitely fibrinolytic. Of the remaining nine strains, eight caused only partial dissolution in 24 hours and one left the clot unaffected.

The percentage of fibrinolytic cultures among the strains of beta hemolytic streptococci isolated in Puerto Rico is of the same magnitude obtained by others working with hemolytic streptococci isolated in temperate climates[88, 86, 39, 38, 32, 50, 84, 80] and by Davis and Guzdar[20] with strains isolated from normal throats in Hong Kong.

Of the 88 group A, 49 group C and 31 group G strains studied; 86, 44, and 27 respectively were fibrinolytic. Of the two group B strains obtained from human sources 1 was fibrinolytic (M 85) and the other (S 5) non-fibrinolytic. The 18 group B strains of bovine origin were non-fibrinolytic.

Table XI also illustrates another important point; namely, the greater fibrinolytic power of cultures of group A streptococci when compared with cultures of group C and G strains. In order to substantiate these findings we tested a number of recently isolated strains from the throats of human beings and normal rhesus monkeys. The cultures were grown in the same medium (glucose neopeptone blood broth) and tested at the same time. Table XII shows the results obtained.

The hemolytic streptococci isolated from the throats of monkeys were also tested for their ability to dissolve rhesus plasma clot. They caused only partial lysis of the monkey plasma in marked contrast to their active fibrinolytic activity against human plasma clot. This strongly suggested that we were dealing with streptococci of ultimate human origin. Seegal et al.[73] had a similar experience with strains isolated from the throats of normal rhesus monkeys in New York.

The results summarized in tables XI and XII show conclusively that cultures of group A strains, when considered as a group, were more strongly fibrinolytic than those of groups C and G. However, there are individual group C and G strains that produce a potent fibrinolysin and group A strains that produce very weakly fibrinolytic cultures. We have occasionally isolated weakly fibrinolytic hemolytic streptococci from human pathological sources and strongly fibrinolytic strains from normal throats, but this is the exception rather than the rule. In this connection we share Dr. Tillett's opinion when he says: "As an arbitrary test for the separation of human pathogenic strains from innocuous ones, the determination of

fibrinolysis is a helpful procedure but is not necessarily conclusive in every instance."

Group A streptococci are the cause of many diseases in man. Human group C and group G strains were found in this work and have been found by others occasionally associated with pathological processes in human beings, but usually they are inhabitants of the normal throat. As will be seen in the section on virulence, our group A strains had, in general, a greater pathogenicity for mice than the C and G strains. Therefore fibrinolysin seems to be associated with and appears to be at least one of the factors concerned in the pathogenesis of beta hemolytic streptococci.

We have not been able to detect any definite relationship between the ability of hemolytic streptococci to produce an erythrogenic exotoxin and fibrinolytic activity. We have tested many times Dochez N. Y. 5 scarlet fever strain, known to be a strong toxin producer, against susceptible plasma from different individuals. This strain has proved to be non-fibrinolytic in all cases.

We have not detected any definite relationship between fibrinolysis and fermentation reaction. Evans[27] has reported that lactose-positive, salicin-negative scarlet fever strains produce a weak fibrinolysin.

Some of the strains in our collection have shown a diminution of their lytic activity after prolonged cultivation on blood agar. Others have been found, under similar conditions, to retain their fibrinolytic power after many months.

There has been some confusion in the literature concerning the fibrinolytic properties of group C streptococci. This appears to have been due to the failure on the part of some investigators to recognize the non-fibrinolytic character (human plasma) of group C strains of ultimate animal origin (i.e., *Str. equi* of horse strangles). We have tested our "human group C" strains from time to time and have found them to have retained, in general, their fibrinolytic properties.

As already stated, a susceptible plasma clot from the same person was used throughout this work. This necessitated a considerable number of bleedings which resulted in loss of time and material and inconvenience to the donor. Therefore we decided to search for a method to preserve plasma so that its ability to clot upon the addition of calcium chloride would be retained at the same time that its susceptibility to lysis would remain unaffected. This was accomplished by the following method[70]:

Human blood from a susceptible person was obtained and distributed in 5 c.c. amounts into small glass bottles, each containing 10 mg. of potassium oxalate in the form of the dry power. After mixing well by gentle shaking, the plasma was separated by centrifugation. The susceptibility of the clot produced with this plasma to the lytic action of hemolytic streptococci was tested, employing the original technic of Tillett and Garner.*[86]

With this information as an initial control on the original plasma the process of preservation was then carried out. The fresh plasma was distributed in 0.3 c.c. amounts into special all glass containers of 5 c.c. capacity.** The plasma was then dehydrated by the Cryochem process using the degassing-self-freezing procedure described by Flosdorf and Mudd.[31] The material was processed for 22 hours at the end of which the tubes were sealed under the original vacuum. The dehydrated product was stored in the ice box.

Some difficulty was encountered in freezing the plasma with the degassing-self-freezing procedure. The dehydrated plasma appeared porous or slightly gelatinous. In localities where dry ice can be obtained, initial freezing with it will greatly simplify the procedure and improve the solubility of the final product.

To perform the fibrinolytic tests with this dehydrated material enough distilled water was added to each tube to restore the original volume. Solubility was accomplished readily by gentle shaking. Samples of this preserved plasma were tested at monthly intervals for its ability to form a firm clot and for its susceptibility to lysis. Similar technic and reagents as employed for testing the fresh plasma were used in all tests. The last sample tested (stored for 8 months in the ice box) clotted in 10 minutes, and dissolution was complete in 18 minutes.

VIRULENCE

Virulence tests were made in two separate series. In the first, the animals were inoculated with the streptococci shortly after isolation. In the second, transplants from stock cultures were used.

First series. At the beginning tryptic digest broth was employed to grow the streptococci. Later glucose neopeptone broth was used. Good growth was obtained in both media. The organisms were cultured for twenty hours at 37° C. in 5 c.c. of medium. The tubes were

* Strain Co. of hemolytic streptococcus was used.

** Catalog No. 112—Cryochem Apparatus (Flosdorf-Mudd). F. J. Stokes Machine Co. of Philadelphia. A model No. 101 Cryochem apparatus was used.

shaken thoroughly in order to obtain an even suspension and mice inoculated intraperitoneally with 0.5 c.c. of culture. One c.c. tuberculin syringes and ½ inch 26 gauge needles were used.

The mice available at the time of the primary isolation of the strains varied. For this reason animals of a standard weight and age were not utilized in this series. The weight ranged from 18 to 28 gms. and the age from 6 to 10 weeks. However, most mice were about six weeks old and weighed about 20 gms.

The animals were kept on a diet consisting of Purina dog chow and water. Those showing no symptoms two weeks after inoculation were killed. Autopsies were not done in some of these cases. Animals that looked sick were allowed to live and were kept under observation for a period of 10 weeks; those that died during this period and most of the survivors were autopsied. Animals which died within two weeks after inoculation were autopsied and a gross examination of the different organs carried out. A blood culture was made from the heart blood and smears of peritoneal exudate and of pus from abscesses were examined. In many instances the pus and peritoneal exudate were cultured. When this was done hemolytic streptococci were always recovered.

In some instances the animals died during the night and when found the next morning showed advanced postmortem decomposition. Smears of the peritoneal exudate contained large numbers of Gram positive and Gram negative rods together with the cocci. The former organisms spread all over the plate when cultures were made and recovery of the streptococci was impossible. Under these conditions the heart's blood was usually contaminated and the growth of the streptococcus was inhibited in the blood cultures. To obviate this difficulty, either the time of inoculation was changed (for example, inoculations were made early in the morning or late in the afternoon), or a smaller number of organisms were introduced. This was done until conclusive evidence was obtained that the death of the animal was due to the streptococcus injected.

Table XIII gives the distribution of the strains tested among the different groups and the relative virulence of strains of groups A, C and G.

In some instances it was noted that apparently normal animals would reveal, when autopsied two or more weeks after inoculation, the presence of abscesses of one or more of the following organs or tissues: peritoneum, liver, lungs, kidneys, abdominal wall at site of inoculation and lymph nodes. In large number of cases peritoneal

abscesses between the liver and stomach or between the spleen and stomach constituted the only gross abnormality. In some instances these abscesses were so large (22 by 10 mm.) that it was surprising that the animals should have lived from six to ten weeks without prominent symptoms. Some mice remained alive as long as ten weeks, but since most of the animals exhibiting no symptoms were killed fourteen days after inoculation, we are unable to say with certainty how long mice can live under these conditions and whether or not some or all of them may ultimately recover. In order to corroborate these findings a second series of mice was studied.

Second series. Almost all the strains of streptococci utilized had been found to be virulent (i.e., would kill mice in from 1 to 14 days) upon isolation. They were classified as follows: 32 group A, 10 group C, 8 group G, and 2 group B. The group C strains were inoculated into mice seven weeks old weighing from 20–25 gms., and the groups A, B and G strains into six weeks old mice weighing from 18 to 20 mgs. A 20 hour old culture in glucose neopeptone broth was used. In each case 10^{-1} c.c. was given intraperitoneally. All animals that died within two weeks were autopsied. All others were killed two weeks after inoculation and autopsied.

Of 52 mice inoculated 22 showed abscesses at autopsy. In fifteen mice peritoneal abscesses located in the regions between spleen and stomach or between liver and stomach were present. In ten instances these abscesses constituted the only prominent gross abnormality.

The formation of these abscesses does not bear a definite relationship to the particular group (A, C or G) or the source of the strain (lymphangitis, sore throat, normal throat, etc.). The dosage, the age of the animal, and the virulence of the individual strain at the time of inoculation appear to be the factors most concerned. The route by which the organisms are introduced into the animal must be considered also. Fourteen animals looked normal two weeks after inoculation. Seven showed no gross abnormalities at autopsy. The other seven mice had abscesses ranging from 1 to 18 mm. in diameter and yet showed no visible symptoms. In view of these results it has been difficult for us to establish a criterion for virulence, but it is apparent that the formation of these chronic abscesses in mice about six weeks old and weighing about 20 gms. is an indication of low virulence.

The results obtained in the present work show, in general, that group A streptococci possess a higher virulence for mice than group C and G strains. However, marked variation occurs and individual

group C and G strains may be highly virulent while some group A strains may possess a low virulence or be completely avirulent.

Review of pathological findings in both series. Generalized peritonitis has been an unusual finding among these animals having been present only in a minority of them. In others there was formation of small abscesses on the peritoneum covering the mesentery and the intestines. In the majority, the inflammatory changes involved the upper abdomen, so that the stomach, pancreas and adjacent portions of the liver and spleen would be found glued to one another by a pale yellow or whitish membrane. In most of these cases, upon trying to free these organs for more exact observations, an abscess would be ruptured, and it would be found to have developed between some of these organs. Some of the animals had fairly large abscesses within the liver.

"Microscopically* there was granulation tissue infiltrated with various types of inflammatory cells, partially covering the above mentioned organs. The pancreas usually showed more or less infiltration of the stroma by the same type of cells, usually monocytes, plasma cells, lymphocytes and polymorphonuclears. The regional lymph glands were enlarged and presented increased numbers of phagocytes within the sinuses and enlarged reticulum cells throughout the lymphoid tissue. Large groups of cocci could be seen in the pus filling the abscesses."

In order to find out if the virulence of the streptococci isolated in Puerto Rico could be enhanced by animal passage six strains were tested. Three had a rather good and the others a poor initial virulence. An 18 to 20 hour old neopeptone glucose blood broth culture was used. Table XIV shows the results of the experiment.

It is interesting to note the relatively high mouse virulence of the group G (M 45) and group C (M 89) strains and their association with severe attacks of sore throat.

The results show that there are strains of hemolytic streptococci isolated in Puerto Rico whose virulence for mice can be considerably exalted by mouse-to-mouse passage as might be expected. It is apparent that this can be accomplished with greater ease with those strains possessing good initial virulence. Since only seven passages were made we cannot say to what extent the virulence of the strains tested could be enhanced by this method.

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* We are greatly indebted to Dr. E. Koppisch, Head of the Department of Pathology of the School of Tropical Medicine at San Juan, for his advice and for the résumé of the microscopical findings.

It has been very difficult to compare the findings concerning the virulence of the hemolytic streptococcus strains isolated in Puerto Rico and that of hemolytic streptococci isolated in temperate regions and studied by others. The main reason for this has been the difference in materials and methods employed and the different criteria for virulence adopted by the various investigators.

Williams[109] tested 75 strains, isolated in the United States, representing different pathological conditions (47 from scarlet fever, 8 from erysipelas, 3 from measles, 17 from other sources). In view of their source it is justified to assume that the vast majority, if not all, of these strains belonged to Lancefield's group A. Williams found that about 50 per cent of her strains killed mice within five days when the animals were inoculated with 0.5 c.c. of an 18 hour broth culture. Sixty per cent of the 95 group A strains tested by us killed mice within four days when a similar dose was utilized. These two percentages, considering the numerous factors affecting this test, show very good agreement.

The opinion held by many that hemolytic streptococci isolated in the tropics possess a lower virulence than strains isolated in temperate climates has been based mainly on clinical observations, and such factors as possible differences in the susceptibility of the host and environmental conditions have been frequently ignored. Only when a considerable number of strains from similar sources, both from tropical and temperate regions, are tested under similar conditions will rejection or acceptance of this idea be justified.

There seems to be no close relationship between virulence and the production of an erythrogenic toxin. We have encountered strains from pathological conditions which do not produce an exotoxin *in vitro* under the conditions of the experiment and, on the other hand, toxigenic strains have been occasionally obtained from normal throats.

Others working with strains isolated in temperate regions have made similar observations.[93, 17, 90, 104]

It must be noted, however, that the vast majority of group A strains (commonly associated with pathological conditions in man) produce a skin exotoxin in marked contrast to the inability of "human group C" and group G strains (only occasionally associated with pathologic conditions in human beings) to produce an erythrogenic exotoxin.

No relationship has been found between the production of hemolysis and virulence. Group C and G strains have been shown to be as

strongly hemolytic, and in some instances more strongly so, as group A streptococci. Whether there exists any inherent difference between the hemolysin produced by these groups has not been definitely settled and awaits further investigation.

SUMMARY AND CONCLUSIONS

A study has been made of a large series of strains of beta hemolytic streptococci all of which were isolated in the tropical island of Puerto Rico. The cultures were obtained from a variety of sources such as normal and pathologic human throats, the throats of normal rhesus monkeys, abscesses, lymphangitis, septicemia, etc., and subsequently grouped according to the Lancefield classification. Their hemolytic properties, fermentation reactions, reduction of methylene blue, hydrolysis of sodium hippurate, final acidity in glucose broth, fibrinolytic power, ability to produce an erythrogenic toxin, and their virulence for mice have been tested and the results compared and tabulated. The main objective was to determine whether hemolytic streptococci of tropical origin differed in their biological and immunological reactions as well as in their pathogenicity for mice from strains isolated from similar sources in temperate regions and studied by other workers.

A method for culturing streptococci from pustules and from other skin lesions has been described. The fact that failure to adhere to it results in an erroneous bacteriological diagnosis in many instances has been strongly emphasized.

The incidence of beta hemolytic streptococci in normal throats has been found to be from four to six per cent. Seasonal variation must be slight if it occurs. This has been contrasted with the high incidence and marked seasonal variation existing in temperate regions.

In a group of 416 individuals representative of the general population, irrespective of the condition of the throat or of any previous history of throat trouble, single throat cultures revealed the presence of beta hemolytic streptococci in 9 per cent. This is in contrast with the 20 to 29 per cent incidence obtained from the throats of persons suffering from recurrent attacks of lymphangitis when single and serial cultures were taken respectively.

From persons with a history of recent throat trouble beta hemolytic streptococci were cultured in 30 per cent.

Attention has been directed to the prevalence of tonsillitis in Puerto Rico. From 100 pairs of excised tonsils examined previously

and from 14 pairs cultured during this study the beta hemolytic streptococcus was isolated in 33 and 28 per cent respectively.

The throats of 172 apparently normal rhesus monkeys were examined soon after their arrival from Calcutta, via New York, and beta hemolytic streptococci were cultured in 12.7 per cent. The biological reactions of these organisms strongly suggested that they were of probable human origin. One of these monkeys died of a septicemia caused by a group A hemolytic streptococcus which produced a viridans appearance on blood agar with the primary cultures.

The association of beta hemolytic streptococci with the initial attack of recurrent lymphangitis is striking. Cultures taken from small lesions on the affected limb, during the acute attack, in 28 cases of lymphangitis revealed the presence of hemolytic streptococci in every instance.

Material from 130 cows with mastitis was studied; beta hemolytic streptococci were not encountered. *Streptococcus agalactiae* was cultured from the great majority of the cases.

Hemolytic *Staphylococcus aureus* and hemolytic streptococci were sometimes found associated in the tissues. The strains of streptococci from such sources exhibited atypical biological properties.

One hundred and seventy-nine of the strains isolated in Puerto Rico were classified by the Lancefield's precipitin test. Some group G and group C strains have been found associated with pathological conditions. A trehalose negative, sorbitol positive, group C streptococcus was found to be definitely associated with a severe attack of sore throat. A group B double-zone hemolytic streptococcus, isolated during life from the blood of a fatal case of puerperal spesis, was described. The distribution of strains isolated in Puerto Rico among Lancefield's groups is the same, in general, as that reported by others working with strains isolated from similar sources in temperate regions.

Group H streptococci were obtained in pure culture from the blood of two cases of rheumatoid arthritis and their biological properties were studied.

The importance of the composition of the medium in the demonstration of soluble hemolysins has been pointed out. When the group A strains were grown in tryptic digest broth they gave a supernatant that was non-hemolytic after reduction with sodium hydrosulphite. However, many of the group C and G strains gave a hemolytic supernatant after reduction when grown in this same

medium. On the other hand 50 per cent of the group A strains gave a hemolytic supernatant after reduction when grown in streptolysin broth. Forty-five per cent of 22 group A strains isolated in temperate regions, included for comparison, produced a supernatant hemolytic after reduction when grown in streptolysin broth. The plurality of streptococcal hemolysin has been briefly discussed.

The production of beta hemolysis in blood agar plates by the strains studied showed a remarkable constancy. This was also true of the fermentation reactions.

The distribution according to Holman's classification was similar to that reported by others with strains isolated from similar sources in temperate climates. The majority of group G strains were *Str. equi* (Holman) and most of the human group C strains tested were non-lactose fermenters. The vast majority of group A strains were *Str. pyogenes* (Holman). All except three of the 175 strains from human sources examined fermented trehalose and not sorbitol. The composition of the medium rather than the mere presence of abundant growth seems to be the important factor affecting fermentation reactions.

Only three (4.3 per cent) of the seventy group A strains tested reduced methylene blue in milk. All of the twenty group B strains (two from human and eighteen from bovine sources) failed to decolorize the dye. Thirty-three (75 per cent) of the 44 human group C strains and 18 (78 per cent) of the twenty-three group G strains reduced methylene blue.

All the strains from human sources, except two, failed to hydrolyze sodium hippurate. The two exceptions were group B strains probably of ultimate bovine origin.

The group A, C and G strains gave an average final pH in glucose broth of 5.2, 5.2, and 5.0 respectively. The group B strains gave an average final pH of 4.7. The variation of the final pH values among the different strains is great and the result obtained with this test is not reliable as a criterion for the classification of individual strains.

The ability of the group A strains to produce a skin exotoxin was comparable to that possessed by group A strains isolated in temperate regions. Seventy-six per cent of the 75 group A strains tested produced an erythrogenic exotoxin.

All the 26 group A strains from lymphangitis produced a skin exotoxin. The findings of Coffey, showing that among fourteen of these twenty-six strains representatives of five different toxin groups were found, have been cited.

Ninety-five per cent of the 173 strains of beta hemolytic streptococci from human sources were fibrinolytic against susceptible human plasma clot. When considered as a whole, group A strains were found to be more strongly fibrinolytic than group C and G strains.

We have not been able to detect any definite relationship between fibrinolysis and the other biological properties studied. There seems to be, in general, some relationship between fibrinolysis and pathogenicity.

A method for the preservation of oxalated human plasma for fibrinolytic tests has been described. Plasma subjected to this process has retained all of the desired characteristics for the fibrinolytic test for a period of eight months.

Group A strains, in general, have been found to be somewhat more virulent for mice than human group C or group G strains.

The production of a chronic streptococcus infection in mice by the intraperitoneal injection of the germs has been followed. In this connection it has been interesting to note an apparent hepatropism (localization of infection in the peritoneum in the region between the liver, stomach and spleen, or the production of abscesses within the liver itself) of many strains.

Due to the differences in materials, methods, and dosage employed and to the different criteria for virulence adopted by different investigators, it has been very difficult to compare results with those of others working with hemolytic streptococci isolated in temperate regions. However, the results obtained by Williams with 75 strains isolated in the United States and representing different pathological conditions, which she tested by similar methods and with the same dose as that utilized in this study, are in very close agreement.

The opinion held by many that hemolytic streptococci isolated in the tropics possess a lower virulence than strains isolated in temperate climates has been based mainly on clinical observation, and such factors as possible differences in the susceptibility of the host and other environmental conditions have been frequently ignored.

Aside from the lower incidence of hemolytic streptococci in the normal throat of human beings in the tropical island of Puerto Rico, as compared to the high incidence obtaining in the throat of normal people in temperate regions, we have not been able to detect, so far, any difference between the biology of strains of beta hemolytic streptococci obtained in Puerto Rico and those isolated elsewhere that would help to explain the relative rarity and comparative

mildness of some streptococcal diseases, such as scarlet fever and rheumatic fever (?), on this tropical island as contrasted with the much higher incidence and severity of the same diseases occurring in temperate climates.

BIBLIOGRAPHY

1. Adamson, H. G. 1916 Streptococcic and staphylococcic eruptions, The Clinical Journal, 45:113.
2. Adamson, H. G. 1937 On the bacteriology of *Pemphigus neonatorum*, and a suggestion for further investigation by a different method of cultural proceeding, Brit. J. Dermat., 49:93.
3. Avery, R. C. 1929 Differentiation of hemolytic streptococci of human and of dairy origin by methylene blue (methylthionine chloride) tolerance and final acidity, J. Exp. Med., 50:463.
4. Ayers, S. H. and Rupp, P. 1922 Differentiation of hemolytic streptococci from human and bovine sources by the hydrolysis of sodium hippurate, J. Infect. Dis., 30:388.
5. Blake, F. G. 1917 The classification of streptococci, J. Med. Res., 36:99.
6. Blake, F. G. 1917 The classification of streptococci, J. Exp. Med., 36:67.
7. Bliss, E. A. 1937 Studies on minute hemolytic streptococci. III. Serological differentiation, J. Bact., 33:625.
8. Bliss, E. A. 1938 Constituent of peptone broth as a cause of cross reactions with antisera prepared against group "C" (Lancefield) hemolytic streptococci, J. Immunol., 34:337.
9. Bourn, J. M., Carpenter, H. M. and McComb, E. 1933 *Streptococcus hemolyticus* (beta) in the presence of acute minor respiratory infection, Amer. J. Hyg., 17:761.
10. Brown, J. H. 1934 Double-zone beta hemolytic streptococci, J. Bact., 27:104.
11. Brown, J. H. 1937 Appearance of double-zone beta hemolytic streptococci in blood agar, J. Bact., 34:35.
12. Brown, J. H. 1939 Double-zone beta hemolytic streptococci; their cultural characteristics, serological grouping, occurrence and pathogenic significance, J. Bact., 37:133.
13. Burky, E. L. and Smillie, W. G. 1929 Nasopharyngeal flora in health and during respiratory disease in isolated communities in Alabama and Labrador, J. Exp. Med., 50:643.
14. Clawson, B. I. 1920 Varieties of streptococci with special reference to constancy, J. Infect. Dis., 26:93.
15. Coburn, A. F. 1931 The Factor of Infection in the Rheumatic State. The Williams and Wilkins Company, Baltimore.

16. Coffey, J. M. and Foley, G. E. 1937 An improved medium for the demonstration of hydrolysis of sodium hippurate by streptococci, Amer. J. Pub. Health, 27:972.
17. Coffey, J. M. and Foley, G. E. 1938 Further observations on the toxigenic properties of hemolytic streptococci, J. Immunol., 35:121.
18. Colebrook, L. 1936 The prevention of puerperal sepsis, J. Obst. and Gynec. Brit. Emp. 43:691.
19. Cougdon, P. M. 1935 Streptococcal infection in childbirth and septic abortion. Source of infection and grouping of hemolytic strains, Lancet, 2:1287.
20. Davis, L. J. and Guzdar, J. S. 1936 The serological, toxigenic and biochemical reactions of streptococci from the throats of Hong Kong Chinese, J. Path. and Bact., 43:197.
21. Dawson, M. H., Hobby, G. L. and Olmstead, M. 1938 Variation in the hemolytic streptococci, J. Infect. Dis., 62:138.
22. Dick, G. F. and Dick, G. H. 1924 Skin test for susceptibility to scarlet fever, J. A. M. A., 82:265.
23. Dochez, A. R., Shibley, G. S. and Mills, K. C. 1930 Studies on the common cold, IV. Experimental transmission of the common cold to anthropoid apes and human beings by means of a filtrable agent, J. Exp. Med., 52:701.
24. Edwards, P. R. 1932 The biochemical characters of human and animal strains of hemolytic streptococci, J. Bact., 23:259.
25. Edwards, P. R. and Broh-Kahn, R. 1934 Hydrolysis of sodium hippurate, J. Bact., 27:535.
26. Evans, A. C. 1936 Studies on hemolytic streptococci, III. *Streptococcus equi* and related strains, J. Bact., 32:541.
27. Evans, A. C. 1937 Studies on hemolytic streptococci. IV. *Streptococcus scarlatinae*, J. Bact. 34:21.
28. Evans, A. C. 1938 Studies on hemolytic streptococci, V. The characteristics of human and animal strains of groups A and C, J. Bact., 36:133.
29. Fisk, E. and Burky, E. 1922 A study on the classification of streptococci, J. Infect. Dis., 30:128.
30. Floch, H. 1938 Etudes de souches de streptocoques isolées de cas de lymphangite endémique des pays chauds. Bull. Soc. Path. exot., 31:888.
31. Flosdorf, E. W. and Mudd, S. 1938 An improved procedure and apparatus for preservation of sera, microorganisms and other substances, The Cryochem process, J. Immunol., 34:469.
32. Fraser, F. H. and Madison, R. R. 1935 Fibrinolytic titer of scarlatinal streptococci, Proc. Soc. Exp. Biol. and Med., 33:307.
33. Fry, R. M. 1938 Fatal infections by hemolytic streptococcus group "B", Lancet 1:199.

34. Fuller, A. T. 1938 The formamide method for the extraction of polysaccharides from hemolytic streptococci, Brit. J. Exp. Path., 19:130.
35. Fuller, A. T. and Maxted, W. R. 1939 The production of hemolysin and peroxide formation by hemolytic streptococci in relation to the non-hemolytic variants of group A, J. Path. and Bact., 49:83.
36. Grace, A. W. and Grace, F. B. 1931 Researches in British Guiana: London School of Hygiene and Tropical Medicine (No. 3 of the memoir series).
37. Griffith, F. 1927 Types of hemolytic streptococci in relation to scarlet fever (second report), J. Hyg., 26:363.
38. Hadfield, G., Magee, V. and Perry, C. B. 1934 The lysis of fibrin by streptococci: Its application to the problems of rheumatic infection in children, Lancet, 1:834.
39. Hare, R. and Colebrook, L. 1934 The biochemical reactions of hemolytic streptococci from the vagina of febrile and afebrile parturient women, J. Path. and Bact., 39:429.
40. Hare, R. 1935 The classification of hemolytic streptococci from the nose and throat of normal human beings by means of precipitin and biochemical tests, J. Path. and Bact., 41:499.
41. Personal communication.
42. Hechler, M. C. and Farrell, M. A. 1938 A study of hemolytic streptococci with reference to the correlation between their group specific substance and biochemical characteristics, J. Infect. Dis., 63:225.
43. Hodge, B. E. and Swift, H. F. 1933 Varying hemolytic and constant combining capacity of streptolysins; influence on testing for antistreptolysins, J. Exp. Med., 58:277.
44. Holman, W. L. 1916 The classification of streptococci, J. Med. Res., 34:377.
45. Howell, K. M. 1924 Effect of brief animal passage on two strains of streptococcus, J. Infect. Dis., 34:117.
46. Jones, F. S. 1919 The streptococci of equines, J. Exp. Med., 30:159.
47. Kirbride, M. B. and Wheeler, M. W. 1924–25 Reactions induced by intracutaneous injections of toxins of streptococci from scarlet fever, Proc. Soc. Exp. Biol. and Med., 22:86.
48. Klimmer, M. and Haupt, H. 1926 Beitrag zur Trennung verschiedener tierpathogener un saprophytischer Streptokokken (des *Streptococcus agalactiae, Str. lacticus, Str. equi, Str. abortus equi* und des *Str. pyogenes equi*), Centrbl. f. Bakt. Orig., 101:126.
49. Klimmer, M., Haput, H. and Roots, E. 1928 Zur Trennung einiger in der Milch verkommender Streptokokken mit besonderer Berücksichtigung der Isolierung des *Str. agalactiae* Gillebeau, Centrbl. f. Bakt. Orig., 107:206.

50. Kodama, T. 1936 Studies on the toxic fractions of hemolytic streptococci. II. The classification of hemolytic and the specific toxigenic properties of group A streptococci, Kitasato Arch. Exp. Med., 13:217.
51. Kodama, T. 1937 Grouping of hemolytic streptococci from normal throats by means of serologic, staining and hemolysin-restriction tests, Kitasato Arch. Exp. Med., 14:29.
52. Krumwiede, E. and Kuttner, A. G. 1938 Growth inhibitory substance for influenza group of organisms in blood of various animal species; use of blood of various animals as selective medium for detection of hemolytic streptococci in throat cultures, J. Exp. Med., 67:429.
53. Lancefield, R. C. 1928 The antigenic complex of *Streptococcus hemolyticus*. I. Demonstration of a type-specific substance in extracts of *Streptococcus hemolyticus*, J. Exp. Med., 47:91.
54. Lancefield, R. C. 1933 A serological differentiation of human and other groups of hemolytic streptococci, J. Exp. Med., 57:571.
55. Lancefield, R. C. and Hare, R. 1935 Serological differentiation of pathogenic and non-pathogenic strains of hemolytic streptococci from parturient women, J. Exp. Med., 61:335.
56. Little, R. B. 1938 Significance of human double-zone beta hemolytic streptococci in udder of cow, J. Exp. Med., 68:905.
57. Long, P. H. and Bliss, E. A. 1934 Studies upon minute hemolytic streptococci. I. The isolation and cultural characteristics of minute beta hemolytic streptococci, J. Exp. Med., 60:619.
58. Long, P. H. Bliss, E. A. and Walcott, C. F. 1934 Studies upon minute hemolytic streptococci. II. The distribution of minute hemolytic streptococci in normal and diseased human beings, J. Exp. Med., 60:633.
59. Milam, D. F. and Smillie, W. G. 1931 Bacteriological study of "colds" on isolated tropical island (St. John, United States Virgin Islands, West Indies), J. Exp. Med., 53:733.
60. Minnet, F. C. and Stableforth, A. W. 1931 Studies on bovine mastitis. IV. The occurrence in mastitis of hemolytic streptococci showing group similarity to *Streptococcus pyogenes* of man, J. Comp. Path. and Therap., 44:114.
61. Morales-Otero, P. 1931 In Coburn's "The Factor of Infection in the Rheumatic State," p. 216. The Williams and Wilkins Company, Baltimore.
62. Morales-Otero, P. 1936 Recurrent tropical lymphangitis with special reference to streptococcus infection, Puerto Rico J. Pub. Health and Trop. Med., 12:1.
63. Morales-Otero, P. and Pomales-Lebrón, A. A bacteriological study of bovine mastitis in Puerto Rico, Unpublished.

64. Ogura, K. 1929 Ueber Drusestreptococcus, mit besonderer Berücksichtung seiner Spezifizität, J. Jap. Soc. Vet. Med., 8:174.
65. Paul, J. H. and Freese, H. L. 1933 An epidemiological and bacteriological study of the "common cold" in an isolated arctic community (Spitzbergen), American J. Hyg., 17:517.
66. Pilot, I. and Davis, D. J. 1919 Hemolytic streptococci in the faucial tonsil and their significance as secondary invaders, J. Infect. Dis., 24:386.
67. Plummer, H. 1934 Fermentation of sorbitol and trehalose by hemolytic streptococci from various sources, J. Bact., 27:465.
68. Plummer, H. 1935 A serological study of hemolytic streptococci, J. Bact., 30:5.
69. Pomales-Lebrón, A. 1936 A bacteriological study of normal throats, pathological throats, and excised tonsils, made in Puerto Rico, Puerto Rico J. Pub. Health and Trop. Med., 11:512.
70. Pomales-Lebrón, A. and Morales-Otero, P. 1939 A method for preservation of oxalated plasma for fibrinolytic tests, Proc. Soc. Exp. Biol. and Med., 41:609.
71. Rolfs, F. O., Trussell, R. E. and Plass, E. D. 1938 Serologic study of streptococci from throat, nose and vagina of ante-partum obstetric patients, Amer. J. Obst. Gynec., 35:1009.
72. Ruiz Nazario, R. and Morales-Otero, P. 1936–37 Dick's test in Puerto Ricans, Puerto Rico J. Pub. Health and Trop. Med., 12:34.
73. Seegal, B. C., Heller, G. and Jablanowitz, J. 1936 Incidence of hemolytic streptococci and pneumococci in the pharyngeal flora of normal rhesus monkeys, Proc. Soc. Exp. Biol. and Med. 34:812.
74. Sharp, W. B. and John, M. B. 1937 Climate and streptococcus rheumatism relationship, J. Infect. Dis., 60:15.
75. Shaw, C. 1937 Serologic grouping and typing of hemolytic streptococci from various human sources, Lancet, 2:1193.
76. Sherman, J. M. 1937 The streptococci, Bacteriol. Rev., 1:3.
77. Smith, F. 1937 Relation of peptone to the production of hemolysin, J. Bact., 34:585.
78. Smith, F. 1937 The influence of various substances and conditions upon streptococcal hemolysins, J. Bact., 34:603.
79. Smith, Th. and Brown, J. H. 1915 A study of streptococci isolated from certain presumably milk-borne epidemics of tonsillitis occurring in Massachusetts in 1913 and 1914, J. Med. Res., 31:455.
80. Stewart, A. B. 1936 The fibrinolytic activity of 269 strains of streptococci, J. Path. and Bact., 43:589.
81. Suárez, J. 1936 Clinical findings in 139 cases of recurrent tropical lymphangitis, Puerto Rico J. Pub. Health and Tropical Med., 12:81.

82. Suárez, J. 1930 A preliminary report on the clinical and bacteriological findings in 60 cases of lymphangitis associated with elephantoid fever in Puerto Rico, Amer. J. Trop. Med., 10:183.
83. Swift, H. F. and Hodge, B. E. 1933 Simply prepared broth for producing hemolytic streptococcal hemotoxin (streptolysin), Proc. Soc. Exp. Biol. and Med., 30:1022.
84. Teiger, P. and Seegal, B. C. 1936 Comparative study of streptococci isolated from throats of residents of New Orleans and New York, J. Bact., 32:631.
85. Thomson, D. and Thomson, R. 1927 Annals of the Pickett-Thomson Research Laboratory, Vol. III, pp. 22–50. The Williams and Wilkins Company, Baltimore.
86. Tillett, W. S. and Garner, R. L. 1933 The fibrinolytic activity of hemolytic streptococci, J. Exp. Med., 58:485.
87. Tillett, W. S., Edwards, L. B. and Garner, R. L. 1934 Fibrinolytic activity of hemolytic streptococci; the development of resistance to fibrinolysis following acute hemolytic streptococcus infections, J. Clin. Invest., 13:47.
88. Tillett, W. S. 1935 The fibrinolytic activity of hemolytic streptococci in relation to the source of strains and to cultural reactions, J. Bact., 29:111.
89. Tillett, W. S. 1938 The fibrinolytic activity of hemolytic streptococci, Bacteriol. Rev. 2:161.
90. Todd, E. W. 1927 Observations on the virulence of hemolytic streptococci, Brit. J. Exp. Path., 8:289.
91. Todd, E. W. 1928 Further observations on the virulence of hemolytic streptococci with special reference to the morphology of the colonies, Brit. J. Exp. Path., 9:1.
92. Todd, E. W. 1928 The conversion of hemolytic streptococci to nonhemolytic forms, J. Exp. Med., 48:493.
93. Todd, E. W. and Lancefield, R. C. 1928 Variants of hemolytic streptococci; their relation to type specific substance, virulence and toxin, J. Exp. Med., 48:751 and 769.
94. Todd, E. W. 1930 Virulence of hemolytic streptococci. I. The influence of oxygen on the production of glossy variants, Brit. J. Exp. Path., 11:368.
95. Todd, E. W. 1932 Antigenic streptococcal hemolysis, J. Exp. Med., 55:267.
96. Todd, E. W. and Hewitt, L. F. 1932 New culture medium for the production of antigenic streptococcal hemolysin, J. Path. and Bact., 35:973.
97. Todd, E. W. 1938 The differentiation of two distinct serological varieties of streptolysin, Streptolysin "O" and Streptolysin "S", J. Path. and Bact., 47:423.

98. Todd, E. W. 1939 The streptolysin of various groups and types of hemolytic streptococci; a serological investigation, J. Hyg., 39:1.
99. Topley, W. C. C. and Wilson, G. S. 1936 The Principles of Bacteriology and Immunity, p. 1548, William Wood and Company, Baltimore.
100. Topley, W. C. C. and Wilson, G. S., Ibid., p. 452.
101. Veldee, M. V. 1932 Standardization of scarlet fever streptococcus antitoxin; method employing ear of white rabbit, Pub. Health Rep. 47:1043.
102. Wadsworth, A. B. 1927 Standard Methods of the Division of Laboratories and Research of the New York State Department of Health, The Williams and Wilkins Company, Baltimore, p. 90.
103. Wadsworth, A. B., Ibid., p. 88.
104. Ward, H. K. and Champs, L. 1935 Studies on the hemolytic streptococcus of human origin. I. Observations on the virulent attenuated and avirulent variants, J. Exp. Med., 61:515.
105. Weld, J. T. 1934 The toxic properties of serum extracts of hemolytic streptococci, J. Exp. Med., 59:83.
106. Wells, J. R. 1923 The origin of immunity to diphtheria in central and polar Eskimos, Amer. J. Hyg., 18:629.
107. Welsh, A. L. 1934 Specificity of a streptococcus isolated from patients with pemphigus; preliminary report, Arch. Dermat. Syph., 30:611.
108. Williams, A. W., Nevin, M. and Gurley, C. R. 1931 Studies on acute respiratory infections. I. Methods of demonstrating microorganisms, including "filtrable viruses," from upper respiratory tract in "health," in "common colds" and in "influenza" with the object of discovering "common strains," J. Immunol. 6:5.
109. Williams, A. W. 1932 Streptococci in Relation to Man in Health and Disease, p. 36. The Williams and Wilkins Company, Baltimore.

TABLE I

Incidence of Beta Hemolytic Streptococci in the Normal Human Throat in Different Parts of the World

Author	*Place*	*Time of year*	*Incidence**
Pilot & Davis[66]	Chicago		58%
Clawson[14]	Chicago	Summer & Fall	37%
Williams, Nevin & Gurley[108]	New York city		6%
Dochez et al.[23]	New York city		17%
Coburn**[15]	New York city	June 1929	90%
Coburn[15]	New York city	Sept. 1929	60%
Coburn[15]	New York city	March 1930	30%
Morales-Otero**[61]	San Juan, Puerto Rico	June 1929	10%
Morales-Otero[61]	San Juan, Puerto Rico	Sept. 1929	10%
Morales-Otero[61]	San Juan, Puerto Rico	March 1930	4%
Sharp & John[74]	Galveston	June–Sept.	13%
		October–May	42%
Topley & Wilson[99]	Manchester	Whole year	12%
Kodama[51]	Tokyo	Winter	88%
Bourn, Carpenter & McComb[9]	Baltimore	Whole year	9.3%
Davis & Guzdar[20]	Hong Kong	Whole year	8.6%
Burky & Smillie[13]	Alabama		3%
Butler***	Melbourne	May–October 1939	32.8%
Milam & Smillie[59]	St. John (Virgin Islands)	Whole year	3%
Grace & Grace[36]	St. Kitts (Br. West Indies)	Feb.–Nov.	15%
Pomales-Lebrón	Puerto Rico	Whole year	4–6%
Burky & Smillie[13]	Labrador		3%
Wells[106]	Alaska (Central & Polar Eskimos)	Summer	13%
Paul & Freeze[65]	Spitzbergen (Arctic region)	Sept. 25–Aug. 10	0.3%

* Throats in general, but free from definite signs of infection when cultures were taken.

** These studies were made concomitantly by Coburn in New York and Morales-Otero in San Juan. The throats of patients in the medical clinic were cultured. The high incidence and marked fluctuation in New York is in striking contrast with the low incidence and slight fluctuation in Puerto Rico.

*** Personal communication.

TABLE II

Source of Strains

Source	*Number of Strains*
Throats: No symptoms. Negative history of throat trouble. Many had hypertrophic, cryptic or scarred tonsils, a hyperemic pharynx, etc	45
Positive history of throat trouble but no symptoms at the time that cultures were taken	5
Pathological	31
Mild scarlet fever	4
Apparently normal monkeys	22
Operated tonsils	14
Abscesses, chronic discharging lesions and pustules in different parts of the body	26
Lymphangitis: small ulcers, pustules or abscesses of affected limb	28
Septicemia*: puerperal	3
Case of agranulocytosis	1
Rhesus monkey	1
Osteomyelitis	2
Otitis media	3
Mastoiditis	2
Wound (abdominal)	1
Infectious eczematoid dermatitis	1
Intestinal ulcer	1

* Two group "H" strains obtained from the blood of cases of rheumatoid arthritis have also been included in this study.

TABLE III

The Distribution of Strains from Different Sources Among Lancefield's Groups

Group	*Throats*						*Excised Tonsils*	*Lymphangitis (small ulcers or pustules)*	*Abscesses, pustules, wounds, osteomyelitis otitis media*	*Blood*
	Normal	*No history. No symptoms. But some abnormality present: scarred tonsils hyperemic pharynx, etc.*	*Previous history of sore throat but no symptoms when culture was taken*	*Sore throat when culture was taken*		*Mild scarlet fever*				
				Mild	*Severe*					
A	4	7	3	1	6	4	9	26	24	2
B	0	0	1	0	0	0	0	0	0	1
C	14	6	6	2	4	0	5	0	2	0
G	10	8	3	2	1	0	0	2	0	2
Total No. of Strains	28	21	13	5	11	4	14	28	26	5

TABLE IV

Grouping According to the Lancefield Classification of Beta Hemolytic Streptococci Isolated from the Throat in Different Parts of the World

Authors	*Place*	*No. of Strains*	*From Normal Throats*				*Throats in General (but not from definite throat infections)*				*Throats—normal monkeys*				*Unclassified*
			A	*B*	*C*	*G*	*A*	*B*	*C*	*G*	*A*	*B*	*C*	*G*	
Shaw[75]	England	29					9 (31%)	1 (3.4%)	?	1 (3.4%)					18
Davis & Guzdar[20]	Hong Kong	78	28 (35.8%)	0	23 (29.4%)	27 (34.6%)									
Hare[40]	England	100					63	5	15	13					4
Rolfs, Trussell & Plass[71]	Iowa	44*					33 (75%)	0	3 (6.8%)	8 (18.2%)					
Kodama[51]	Tokyo	78	38 (48.7%)	2 (2.5%)	18 (23.1%)	20 (25.7%)									
Butler†	Melbourne	70					23 (32.8%)	?	?	?					
Pomales-Lebrón	Puerto Rico	28	4 (14.3%)	0	14 (50%)	10 (35.7%)									
Pomales-Lebrón	Puerto Rico	62					14 (22.6%)	1 (1.6%)	26 (42%)	21 (33.8%)					
Seegal, Heller & Jablanowitz[73]	New York City	28									19 (68%)	0	4 (14%)	5 (18%)	
Pomales-Lebron	Puerto Rico	24									10 (41.7%)	0	9 (37.5%)	5 (20.8%)	

* From the throats of 500 obstetric patients. † Personal communication.

TABLE V

Production of Hemolysin in Tryptic Digest Broth

Strain	*Source*	*Group*	*Tube Hemolysis**		
			W	*S u*	*S r*
L 5	Lymphangitis (small ulcer)	A	++++	—	—
L 6	" " "	A	++++	—	—
L 7	" " "	A	++++	—	—
L 8	" " "	A	++++	—	—
L 9	" " "	A	++++	—	—
L 15	" " "	A	++++	—	—
A 3	Lesion on knee joint	A	+++	—	—
A 4	Pustules all over body	A	++++	—	—
A 5	Deep chronic lesions (face and neck)	A	+++	—	—
A 8	Abscess	A	++++	—	—
A 10	"	A	++++	—	—
A 19	Wound (finger)	A	++++	—	—
T 31	Excised tonsil	A	++++	—	—
T 1	" "	A	++++	—	—
T 4	" "	A	++++	—	—
T 5	" "	A	+++	—	—
T 10	" "	A	+++	—	—
T 11	" "	A	+++	—	—
M 15	Tonsillitis	A	++++	—	—
M 18	Tonsillitis	A	++++	—	—
M 79	Severe throat	A	++++	—	—
M 72	Normal throat	A	++++	—	—
M 90	Normal throat	A	++++	—	—
C.Mo. K	Throat, normal monkey	A	++++	—	—
C.Mo. 37	" " "	A	+++	—	—
C.Mo. 47	" " "	A	++++	—	—
Sc. 1	Throat, mild scarlet fever	A	++++	—	—
Sc. 2	" " " "	A	++++	—	—
Sc. 3	" " " "	A	++++	—	—
S 2	Blood, puerperal sepsis	A	++++	—	—
O 1	Osteomyelitis	A	++++	—	—
O 2	"	A	+++	—	—
O.M. 1	Otitis media	A	++++	—	—
O.M. 2	" "	A	++++	—	—
L 23	Lymphangitis (small ulcer)	A	++++	++	—

TABLE V (*Continued*)

Production of Hemolysin in Tryptic Digest Broth

Strain	*Source*	*Grp.*	*Tube Hemolysis**		
			W	*S u*	*S r*
L 24	Lymphangitis (small ulcer)	A	++++	+	—
M 69	Normal throat	A	++++	+++	—
M 65	Chronic tonsillitis	A	++++	+++	—
M 13	Normal throat	C	+++	—	—
M 57	" "	C	+++	—	—
T 13	Excised tonsil	C	++++	—	—
T 6	" "	C	+++	—	—
T 15	" "	C	+++	++	—
M 43	Sore throat	C	++++	++++	±
M 29	Normal throat	C	++++	+++	±
M 50	" "	C	++++	++	±
M 10	Tonsillitis	C	++++	—	++++
M 11	Mild sore throat	C	++++	—	++
M 14	" " "	C	+++	—	+++
M 27	Normal throat	C	++++	±	++++
M 32	Tonsillitis	C	+++	—	+++
M 41	Normal throat	C	+++	—	+
M 49	" "	C	++++	—	++++
M 66	Throat, no symptoms	C	++++	—	++++
M 75	" " "	C	+++	—	+++
C.Mo. 1	Throat, normal monkey	C	++++	—	++++
C.Mo. 8	" " "	C	+	—	+
M 68	Sore throat	G	+	—	+++
S 3	Septicemia (agranulocytic angina)	G	++++	—	—
S 4	Blood, puerperal fever	G	++++	++	—
M 15	Normal throat	G	++++	—	+
M 21	" "	G	++++	—	+
M 30	Chronic tonsillitis	G	++++	—	++
M 31	" "	G	++++	—	+++
M 34	Normal throat	G	+++	—	+++
M 35	Sore throat	G	+++	—	+++
M 39	" "	G	+++	—	+++

* W = Whole culture.
S u = Unreduced supernatant.
S r = Reduced supernatant.

TABLE VI

Production of Hemolysin in Streptolysin Broth

Strain	*Grp.*	*Tube Hemolysis*		
		W	*S u*	*S r*
L 5	A	++++	+	++++
L 7	A	++++	++	+++
L 8	A	++++	+	—
L 9	A	++++	++	—
L 26	A	++++	—	—
L 24	A	++++	—	++++
L 27	A	++++	+	++++
L 23	A	++++	—	++++
L 18	A	++++	±	++++
L 19	A	++++	—	++++
L 12	A	++++	—	—
L 17	A	++++	—	++
L 22	A	++++	±	+++
L 20	A	++++	±	++++
L 11	A	++++	++	—
M 85	A	++++	±	—
A 15	A	++++	—	++++
L 28	A	++++	—	++++
A 22	A	++++	—	—
A 17	A	++++	+	+++
A 23	A	++++	—	++++
A 19	A	++++	—	—
A 8	A	++++	++	—
A 3	A	++++	—	—
A 24	A	++++	±	—
A 20	A	++++	—	++++
A 18	A	++++	+	++++
A 21	A	++++	++	—
A 5	A	++++	—	—
O.M. 2	A	++++	—	—
S 2	A	++++	++	—
O 2	A	++++	—	++++
Ma. 2	A	++++	++	—
O.M. 1	A	++++	±	—
Sc. 1	A	++++	—	—
O.M. 3	A	++++	+	++++
M 15	A	++++	—	—
M 72	A	++++	—	—
M 61	A	++++	—	+++
M 23	A	++++	—	++
M 79	A	++++	+++	±
M 12	A	++++	+	++++
M 52	A	++++	+	—

Strain	*Grp.*	*Tube Hemolysis*		
		W	*S u*	*S r*
M 48	A	++++	+	—
M 69	A	++++	++	++++
M 65	A	++++	+	++++
M 76	A	++++	+	++++
T 30	A	++++	—	—
T 11	A	++++	+	±
T 14	A	++++	—	±
T 31	A	++++	±	+
T 4	A	++++	±	++++
C.Mo. 17	A	++++	—	—
C.Mo. 5	A	++++	—	—
C.Mo. 118	A	++++	±	—
C.Mo. 71	A	++++	+	—
C.Mo. 37	A	++++	±	++++
C.Mo. 68	A	++++	—	++++
C.Mo. 47	A	++++	+	—
C.Mo. 157	A	++++	++	++++
C.Mo. K	A	++++	—	++++
M 86	B	+++	±	++
M 57	C	++++	—	±
M 50	C	++++	—	±
M 43	C	++++	++	++++
M 71	C	++++	±	++++
M 19	C	++++	—	++++
T 13	C	++++	—	++++
M 37	C	++++	—	++++
M 13	C	++	—	++++
M 38	C	+++	—	+++
M 89	C	++++	±	—
C.Mo. 70	C	++++	—	++++
C.Mo. 2	C	++++	—	++++
C.Mo. 64	C	++++	—	++++
C.Mo. 121	C	++	—	++++
S 3	G	++++	—	++++
S 4	G	++++	—	++++
M 77	G	++++	—	++++
M 39	G	+++	—	++++
M 16	G	++++	—	++++
M 21	G	++++	++	++++
M 56	G	++++	±	++++
M 40	G	++++	±	++++
C.Mo. 4	G	++++	—	++++
C.Mo. 28	G	+++	—	++++

TABLE VII

Final pH in Glucose Broth, Reduction of Methylene Blue and Hydrolysis of Sodium Hippurate

Source	Number of Strains	Grp.	Final pH in glucose broth	Reduction of methylene blue	Hydrolysis of sodium hippurate
Human	1	A	4.3	+++	—
	1	A	4.7	—	—
	1	A	4.8	—	—
	1	A	4.9	—	—
	2	A	5.0	—	—
	1	A	5.1	++++	—
	4	A	5.1	—	—
	13	A	5.2	—	—
	17	A	5.3	—	—
	2	A	5.3	+++	—
	15	A	5.4	—	—
	8	A	5.5	—	—
	3	A	5.6	—	—
	1	A	5.8	—	—
	2	B	4.4	—	+
	1	C	4.7	—	—
	2	C	4.8	++	—
	1	C	4.8	+++	—
	1	C	4.8	++++	—
	1	C	4.9	+	—
	4	C	4.9	+++	—
	1	C	4.9	—	—
	1	C	5.0	±	—
	1	C	5.0	++	—
	3	C	5.0	+++	—
	2	C	5.0	—	—
	1	C	5.1	++	—
	5	C	5.1	+++	—
	1	C	5.1	++++	—
	2	C	5.1	—	—
	1	C	5.2	++	—
	2	C	5.2	+++	—
	1	C	5.2	+	—
	1	C	5.3	±	—
	2	C	5.3	+++	—
Human	1	C	5.3	++++	—
	1	C	5.3	—	—
	1	C	5.4	+++	—
	2	C	5.4	—	—
	1	C	5.5	+++	—
	1	C	5.5	++++	—
	1	C	5.5	—	—
	1	C	5.6	—	—
	1	C	5.7	++++	—
	1	G	4.7	+++	—
	1	G	4.8	+++	—
	1	G	4.8	++++	—
	3	G	4.8	—	—
	1	G	4.9	++	—
	1	G	4.9	+++	—
	1	G	5.0	+	—
	1	G	5.0	++	—
	2	G	5.0	+++	—
	1	G	5.0	++++	—
	1	G	5.1	++	—
	1	G	5.1	+++	—
	1	G	5.1	—	—
	1	G	5.2	+++	—
	1	G	5.2	—	—
	1	G	5.3	+++	—
	1	G	5.3	++++	—
	1	G	5.4	++	—
	1	G	5.4	++++	—
	1	G	5.6	+++	—
Cow (mastitis)	2	B	4.4	—	+
	3	B	4.6	—	+
	6	B	4.7	—	+
	2	B	4.9	—	+
	2	B	5.0	—	+
	2	B	5.2	—	+
	1	B	5.3	—	+

Average final pH in glucose broth:

Group A (70 strains, human).....5.2
Group C (44 strains, human).....5.2
Group G (23 strains, human).....5.0
Group B (18 strains, cow mastitis, 2 strains human source)....4.7

TABLE VIII

Final pH Values in Glucose Broth Reported by Different Authors

Author	*Lancefield's Groups*			
	A	*B*	*C*	*G*
Plummer[68]	5.0–5.2	4.4–4.6	5.0–5.2 (rabbits, horses, guinea pigs)	4.4–4.6 (normal throats, tonsillitis)
Hare*[40]	4.7–5.2	4.5–4.7	4.9–5.3	4.9–5.0
Davis & Guzdar**[20]	28 strains examined. "All 4.8 or higher. Twenty-one, 5.0 or higher."		"Fifty C and G. Only 12 higher than 4.6."	
Hechler & Farrell[42]	4.3–4.9	4.4	4.5–4.8	4.5–4.8
Avery***[3]	5.0–5.2	4.4–4.5		
Lancefield[54]	23 strains examined. One 4.8. The rest, 5.0–5.2.	4.4–4.6	4.6–4.9 "From a variety of animal sources other than man."	
Teiger & Seegal[84]	5.0–5.4			
Pomales-Lebrón	5.2 (average)	4.7 (average)	5.2 (average)	5.0 (average)

* From the nose and throat of normal persons.

** These authors state: "Of the total 78 strains examined, then, 59 displayed a definite correlation between their serological grouping and their hydrogen ion reaction as determined by the test. In this respect our findings differ from those of Hare whose C and G strains of human origin gave final pH values similar to those of his group A strains."

*** Avery was working with 1. "Human parasitic strains, defined by a final pH range of 5.2 to 5.0 and by failure to reduce methylene blue (1:5000) in milk," and 2. "bovine strains parasitic in the udder. . ." It is believed justified to consider these two groups of strains as belonging to groups A and B.

TABLE IX

Production of Erythrogenic Toxin. Filtrates Tested in the Skin of a Susceptible White Goat

Source	*No. of Strains*	*Group*	*Reaction (average diameter of area of erythema)*
Lymphangitis	19	A	20 mm.
"	1*	A	±
Abscesses, pustules chronic discharging lesions, osteomyelitis, impetigo	11	A	19 mm.
Abscesses (2 strains) meningitis, serous exudate from knee joint	4	A	—
Otitis media	2	A	25 mm.
" "	1	A	—
Osteomyelitis	1	A	20 mm.
"	1	A	±
Blood	1	A	23 mm.
Blood (rhesus monkey)	1	A	20 mm.
Mastoiditis	1	A	20 mm.
"	1	A	—
Tonsillitis	5	A	22 mm.
Excised tonsils	6	A	15 mm.
" "	2	A	—
Throat: no symptoms but (a) previous sore throat or (b) hyperemic pharynx, scarred tonsils, etc.	6	A	24 mm.

* Mixed in tissues with *Staphylococcus aureus*.

TABLE IX (*Continued*)

Production of Erythrogenic Toxin. Filtrates Tested in the Skin of a Susceptible White Goat

Source	*No. of Strains*	*Group*	*Reaction (average diameter of area of erythema)*
Hyperemic pharynx (no symptoms)	1	A	±
Normal throat	1	A	20 mm.
" "	1	A	±
" "	1	A	—
Throats, normal rhesus monkeys	3	A	20 mm.
" " " "	2	A	±
" " " "	3	A	—
Blood	1*	B	—
Throat, no symptoms (history of sore throat)	1	B	—
Tonsillitis, sore throat	9	C	—
Excised tonsils	5	C	—
Throat, no symptoms but (a) previous sore throat or (b) hyperemic pharynx, scarred tonsils, etc.	10	C	—
Hypertrophic septic tonsils, no symptoms	1	C	±
Normal throats	12	C	—
Throats, normal rhesus monkeys	9	C	—
Abdominal wound	1	C	—
Intestinal ulcer	1	C	—
Blood	2	G	—
Tonsillitis, sore throat	5	G	—
Throat, no symptoms but (a) previous sore throat or (b) hyperemic pharynx, scarred tonsils, etc.	8	G	—
Previous mild sore throat	1	G	23 mm.
Normal throats	9	G	—
Throat, normal rhesus monkeys	4	G	—

* Double-zone hemolysis.

TABLE X

Toxigenic Properties of Hemolytic Streptococci Isolated from Cases of Lymphangitis

Strain	*Neutralization of toxin by monovalent antistreptococcus horse sera*			
	No. 165 (scarlet fever)	*No. 337 (erysipelas)*	*No. 32369 (rheumatic fever)*	*No. 32283 (septic sore throat)*
L 5	+	+	—	+
L 6	+	+	—	+
L 7	+	+	—	+
L 8	—	—	—*	—
L 9	+	+	—	+
L 11	—	—	—	+
L 13	nontoxigenic			
L 14	—	—	+	—
L 15	—	—	—	±**
L 16	+	+	—	+
L 17	—	—	—	±**
L 18	+	+	—	+
L 19	—	—	+	—
L 20	+	+	—	+
L 21	nontoxigenic			

* Neutralized by serum of strain No. 165 combined with equal parts of serum of strain No. 32369.

** Neutralized by serum of strain No. 165 combined with equal parts of serum of strain No. 32283.

TABLE XI

Production of Fibrinolysin in the Different Media by Strains of Groups A, C and G

Average Dissolution Time								
Tryptic digest broth			*Glucose neopeptone broth*			*Glucose neopeptone blood broth*		
A	*C*	*G*	*A*	*C*	*G*	*A*	*C*	*G*
3 hrs.	8 hrs.	9 hrs.	1.8 hrs.	3.3 hrs.	2.9 hrs.	1 hr.	2.1 hrs.	3.4 hrs.
(42)*	(14)	(5)	(14)	(12)	(14)	(25)	(18)	(12)

* Figures in parentheses indicate number of strains tested.

TABLE XIV

Exaltation of Virulence by Mouse to Mouse Passage

Strain	*Group*	*Source*	*Initial Virulence**	*Virulence after 7th mouse passage*
M 15	A	Severe sore throat	10^{-3} c.c.	10^{-5} c.c.
M 45	G	Severe sore throat	10^{-2} c.c.	10^{-4} c.c.
M 89	C	Severe sore throat	10^{-3} c.c.	10^{-5} c.c.
A 26	A	Deep chronic lesions of lower limb	0.5 c.c.	10^{-1} c.c.
M 90	A	Asymptomatic hyperemic pharynx	0.5 c.c.	10^{-1} c.c.
M 91	A	Asymptomatic hyperemic pharynx	10^{-1} c.c.	10^{-3} c.c.

* Ability to kill the animal in 4 days or less.

TABLE XII

Fibrinolytic Power of Recently Isolated Strains of Groups A, C and G

Strain	*Source*	*Group*	*Dissolution time*
D.M. 1	Blood, rhesus monkey	A	40 min.
L 28	Lymphangitis (small ulcer)	A	20 min.
A 26	Abscess	A	1 hr.
M 90	Hyperemic pharynx (no symptoms)	A	25 min.
M 91	Hyperemic pharynx (no symptoms)	A	2 hrs.
C.Mo. 5	Throat, normal rhesus monkey	A	25 min.
C.Mo. 17	" " " "	A	23 min.
C.Mo. 37	" " " "	A	18 min.
C.Mo. 47	" " " "	A	54 min.
C.Mo. 68	" " " "	A	1 hr. 45 min.
C.Mo. 71	" " " "	A	1 hr. 16 min.
C.Mo. 118	" " " "	A	45 min.
C.Mo. 157	" " " "	A	42 min.
M 89	Severe sore throat	C	15 min.
M 92	Hyperemic pharynx (no symptoms)	C	1 hr. 30 min.
M 94	Chronic tonsillitis	C	4 hrs.
M 95	Hypertrophic septic tonsils (no symptoms)	C	1 hr. 30 min.
C.Mo. 1	Throat, normal rhesus monkey	C	1 hr. 38 min.
C.Mo. 2	" " " "	C	4 hrs. 30 min.
C.Mo. 8	" " " "	C	2 hrs. 30 min.
C.Mo. 18	" " " "	C	4 hrs. 20 min.
C.Mo. 64	" " " "	C	4 hrs. 30 min.
C.Mo. 70	" " " "	C	25 min.
C.Mo. 121	" " " "	C	1 hr. 15 min.
C.Mo. 159	" " " "	C	2 hrs.
M 82	Normal throat	G	2 hrs. 15 min.
M 83	Mild sore throat	G	5 hrs.
M 87	Chronic tonsillitis	G	50 min.
M 93	Hyperemic pharynx, scarred tonsils (no symptoms)	G	2 hrs.
P.R.Mo. 1	Throat, normal spider monkey	G	15 min.
C.Mo. 4	Throat, normal rhesus monkey	G	5 hrs.
C.Mo. 6	" " " "	G	5 hrs. 20 min.
C.Mo. 28	" " " "	G	5 hrs. 30 min.

Averages: Group A, 50 min.; Group C, 2.6 hrs.; Group G, 3 hrs.

TABLE XIII

*Virulence for Mice of Groups A, C and G Strains**

Group	*Number of strains tested*	*Killed in one to four days*	*Killed in 5 to 14 days*	*Animals still alive after two weeks*
A	95	58 (60%)	10 (10.5%)	27 (29.5%)
C	56	23 (40.5%)	4 (7%)	29 (52.5%)
G	23	11 (45%)	3 (12%)	11 (43%)

* Two group B strains of human origin tested killed mice in 2 to 4 days.