STUDIES ON THE ANAEROBIC STREPTOCOCCUS[1]

I. Certain Biochemical and Immunological Properties of Anaerobic Streptococci

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I. HISTORICAL AND INTRODUCTION

Despite the fact that the anaerobic streptococcus has been studied previously, many of its biochemical and immunological properties have not been investigated. The fact that we, in common with many others, noted puerperal infections which appear to be caused by anaerobic streptococci, has prompted the present investigation of these organisms with a view to determining the conditions under which they initiate infection. It seemed best, in view of the paucity of knowledge concerning the reactions and characteristics of the anaerobic streptococci that the study should proceed, at first, through a consideration of the properties of the organisms from a biochemical and immunological standpoint. From this it was hoped that perhaps certain differences and similarities might be discovered, some of which might be correlated with the virulence or lack of virulence possessed by particular strains, and that such knowledge might be applied in the determination of the pathogenicity of strains isolated at some future time.

The anaerobic streptococci have been reported by various authors (Taylor (1929)) in pure or mixed cultures from the uterus and blood stream in puerperal infections, from pulmonary abscesses, pelvic cellulitis, sinusitis, pleurisy, Ludwig's angina, empyema of the pleura and cerebro-spinal meningitis. White (1933) found them to be present in the vagina of fully 30 to 40 per cent of normal women.

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Veillon in 1893 described a strictly anaerobic coccus isolated from cases of Ludwig's angina, perinephric abscess and bartholinitis. This he designated as *Micrococcus fetidus* because of its property of producing gas and a foul odor in culture media. Rist (1898), Hallé (1898) and Guillemot (1898) isolated similar organisms from other sites. Kronig and Menge, independently, and later in collaboration (1895–1899), also described a strictly anaerobic coccus isolated from the vagina in pregnancy and puerperal infections. They included one group of anaerobic streptococci which produced gas and foul smelling decomposition products. Lewkowicz (1901) isolated from babies' mouths a minute obligate anaerobic coccus which produced neither gas nor foul odor and which he termed *Streptococcus anaerobius micros.* From time to time other strains were described; a notable group mentioned by Schottmüller (1910), was isolated from puerperal infections and produced gas and foul odor only in media containing blood. This organism he called *Streptococcus putridus.*

Prévot in 1925 made the first systematic attempt at classification of the anaerobic streptococci and recognized three groups:

Group "A"—Anaerobic streptococci which produce gas and fetid odor in special media. This group includes *Micrococcus fetidus* of Veillon (1893), *Streptococcus anaerobius* of Kronig (1895) and *Streptococcus putridus* of Schottmüller.

Group "B"—Anaerobic streptococci which produce neither gas nor fetid odor. In this group are placed *Streptococcus micros* of Lewkowicz (1901) and a new organism, *Streptococcus intermedius* isolated by Prévot in 1925.

Group "C"—Anaerobic streptococci of predilection producing neither gas nor odor (*Streptococcus evolutus* of Graf and Wittneben 1907).

Colebrook and Hare (1933) classified the anaerobic streptococci according to colony morphology on blood agar and gas production and recognized four groups:

Group "A"—Opaque colonies without hemolysis—produce gas in liquid media.

Group "B"—Translucent colonies without hemolysis—produce no gas in liquid media.

Group "C"—Coal black colonies—produce gas in liquid media.

Group "D"—Produce hemolysis but appear similar to and may be variants of Groups "A" and "B".

They state that groups "C" and "D" are rare while "A" and "B" are common. Further, they found by agglutination reactions that groups "A" and "B" appear serologically distinct.

All authors agree that laboratory animals are generally refractory to the anaerobic streptococcus. Marwedel and Wehrsig (1915) were able in one case to cause gas-gangrene and death of a guinea-pig within twelve hours after infection with a strain isolated from a fatal war-wound. This strain, however, became rapidly avirulent on artificial cultivation and it is the consensus of opinion that a similar rapid loss of virulence is responsible for the failure of anaerobic streptococci to reproduce infection in laboratory animals. Schottmüller (1910) reports that *S. putridus* is capable of producing a characteristic clinical picture in human beings following septic thrombophlebitis of the parametrial veins in puerperal infections and we have observed many cases similar to the type he describes. As a matter of fact, the occurrence of this picture coupled with the finding of blood cultures positive for anaerobic streptococci first stimulated our interest in this organism and it is generally held that despite the apparent lack of pathogenicity for laboratory animals, the organism is without doubt pathogenic for man.

II. SOURCE OF MATERIAL AND METHODS OF ISOLATION

A. Source of cultures

In this study, cultures of anaerobic streptococci were secured from parturient and post-abortal women by means of a sterile pipette inserted through the cervix into the uterus. In this manner about 1 to 2 ml. of lochia were secured and samples of this material were inoculated into infusion broth and into cooked meat medium which was then sealed with vaseline.[2] The medium was deaerated by boiling prior to inoculation. In addition, 5 per

[2] Cooked meat medium consisted of beef heart infusion broth to which about ¾ inch of ground, cooked meat had been added. The final pH of this medium was 7.5.

cent horse blood agar plates at a pH of 7.6 were inoculated by the streak method and incubated aerobically and anaerobically. Anaerobic plates were placed in Brown jars and incubated for 48 hours at 37°C. At the conclusion of incubation, smears were made of both the aerobic and anaerobic cultures. Sub-culturing of the anaerobic growths was then done so as to isolate pure cultures of obligate anaerobic streptococci. In all cases the pH of the lochia was measured by means of the glass electrode within a few minutes after the material was secured.[3] In many cases where infection of the blood stream with the organism was suspected, blood samples were secured and cultured in the same manner as outlined above. Clinical records of the course of the patient both prior and subsequent to the taking of cultures were kept.

Cultures secured in this manner were kept in cooked meat medium and transferred every three to four days. Stained preparations were made at each transfer to disclose the presence of possible contaminants. In addition, cultures were kept on horse-blood agar plates in the refrigerator and transferred monthly. Periodically, sub-cultures were made from broth to aerobic plates to determine whether or not growth would occur in the presence of atmospheric oxygen tension.

Samples of lochia or blood in which anaerobic streptococci could not be identified were discarded.

B. Occurrence, cultural characteristics and description of anaerobic streptococci isolated

A total of forty cultures were taken from the uterus in the course of this investigation and in twenty-five cases, anaerobic streptococci were isolated either in pure culture or in association with other organisms. The incidence of anaerobic streptococci is therefore about 62.5 per cent, a figure which is considerably higher than that reported by White (1933). It must be remembered in this connection, however, that the great majority of our cultures was secured from women suffering with some form of puerperal or post-abortal infection, whereas White estimated the

[3] The Beckman glass electrode was used to determine the pH in all cases. 2 ml. of the lochia were utilized in pH determinations. Where amounts of lochia were less than 2 ml., distilled water was added to the required volume.

incidence of anaerobic streptococci in the vaginae of normal women. One of our strains was isolated from a woman during an apparently normal puerperium while twenty-four were isolated from patients having a morbid puerperal course. A total of twenty-one blood cultures was taken, only one of which was positive for the anaerobic streptococcus. Five other blood cultures were positive for the aerobic organisms, *Streptococcus hemolyticus* and *Staphylococcus hemolyticus*. Two of the patients from whom hemolytic streptococci were isolated, subsequently died.

On horse blood agar, at the end of forty-eight hours in the Brown jar, all our strains of anaerobic streptococci had a superficial resemblance. Generally speaking, the colonies were small, round, slightly convex, and greyish white in color. The cultures had a peculiar and quite characteristic sweetish-foul odor on this medium. In no case was hemolysis noted, either immediately or after 96 hours, but after storage in the refrigerator for a period of months, small zones of hemolysis were noted surrounding each colony. On sub-culture of these colonies, however, hemolysis did not occur within 96 hours. (For grouping according to colony morphology, see table 1.)

In cooked meat medium, under vaseline seal, the vast majority of the strains required 48 hours for good growth, at the end of which time the organisms were seen to have grown best around the meat at the bottom of the tube. Seven of the strains produced gas, as evidenced by the displacement of the vaseline seal upward. In addition, fifteen of the twenty-six strains produced a very foul, putrid odor (see table 2).

Since obligate anaerobiosis was the *sine qua non* for inclusion of a strain in the series, it goes without saying that none of the organisms grew under aerobic conditions initially. In order to check the tolerance to oxygen, periodic sub-cultures from anaerobic to aerobic media were made. After cultivation over periods varying from six to eight months, four of our strains (see table 1) exhibited tolerance to aerobiosis and grew well on horse blood agar. However, the remaining twenty-two strains which have been cultivated artificially for periods varying from four to twelve months, at the present time show no tendency to grow aerobically.

Microscopically, the organisms comprising the greater percent-

TABLE 1

Colony description

ROUND, CONVEX, TRANSLUCENT, GLOSSY, GREYISH WHITE STRAINS	ROUND, SLIGHTLY CONVEX, TRANSLUCENT, LIGHTER AT EDGES, DULL, GREYISH WHITE STRAINS	ROUND, PUNCTIFORM, TRANSPARENT, EFFUSE, GREY STRAINS
Hod	Odu	Mur
Gow	Odo	Hub
Cob	TerA	Kud
Syl II	TerB	Syl
Tam*	Kas	
Dre	Ase	
Fer	Dau	
Def	Eff	
Mik	Lin	
Rze*		
Myi*		
Myt		
Len*		
13	9	4

* Indicates strains which later exhibited tolerance to aerobic conditions.

TABLE 2

Gas and odor production

96 hour incubation

FOUL ODOR ALONE	GAS ALONE	GAS AND FOUL ODOR	NO GAS OR ODOR
Hub	Mik	Odu	Mur
Hod	Def	Cob	Syl II
TerA		Syl	Gow
TerB		Dau	Eff
Myi		Kud	Lin
Ase			Rze
Kas			Fer
Myt			Odo
Tam			Dre
Len			
10	2	5	9

age of the strains were characteristically small, coccal forms with a tendency to form short chains of from six to eight elements in liquid medium. The gram stain was unreliable since, while

most of the organisms were gram positive, many were negative at times and positive at others, despite the fact that gram controls indicated a technically adequate stain. After prolonged cultivation, a few of the strains formed diphtheroid types. That these were not contaminants was shown by repeated plating from individual colonies. Further, a similar observation has been recorded by Weiss and Mercado (1938) with their strain NiB.

III. EXPERIMENTAL METHODS

A. Biochemical methods

1. The fermentation of trehalose and sorbitol. The medium used was a casein digest broth consisting of 90 ml. beef infusion broth and 10 ml. of casein digest. To 7 ml. of this basic medium in inverted fermentation tubes 0.78 ml. of a 10 per cent solution of trehalose or sorbitol was added. The sugar had been previously dissolved in distilled water and sterilized by filtration through a Berkefeld "V" filter. The basic medium was adjusted to pH 7.6 and sterilized in the autoclave before the addition of the sugar solution. In addition, during the early work on this test, 1 per cent of Andrade's indicator was added to the medium, but when it was determined that the dye frequently inhibited growth, it was omitted in later studies.

The medium described above was inoculated with a standard loopful of the organism and incubated in the Brown jar for a period of ten days at 37°C. At the conclusion of this period the color reactions were noted (in those cases in which indicator had been added) and the pH of the medium was taken by means of a Beckman pH meter. Appropriate positive and negative controls were included in each jar, the positive control being Group E[4] hemolytic streptococcus and the negative control being Group C[5] hemolytic streptococcus. Uninoculated medium incubated with the cultures was used to control possible pH drift in the medium. The final determinations of fermentation were repeated at least twice in each case.

[4] Lancefield strain K129.
[5] Lancefield strain K104.

2. The hydrolysis of sodium hippurate. The test of the hydrolysis of sodium hippurate was made according to the method of Ayers and Rupp (1922). The cultures were grown anaerobically in the Brown jar for four days in a medium consisting of infusion broth to which had been added 1 per cent of sodium hippurate. At the conclusion of the test period, the cultures were centrifugated and to 1 ml. amounts of the clear supernatant were added 0.3, 0.4, and 0.5 ml. respectively of a 12 per cent ferric chloride solution containing concentrated hydrochloric acid in the proportion of 2.5 ml. per liter. Uninoculated sodium hippurate broth incubated for the same period of time was used as a control, and sufficient ferric chloride was used in the test to insure complete clearing of the uninoculated control. The tubes were shaken vigorously, immediately upon the addition of the ferric chloride. In the case of a positive reaction, a heavy precipitate of ferric benzoate, insoluble in an excess of ferric chloride, was formed. If the reaction was negative, the protein and hippurate precipitate formed upon the addition of ferric chloride, redissolved in the excess, leaving a clear solution. Known positive[6] and negative[7] cultures were included as controls in each set of tests.

3. Final pH in 1 per cent glucose broth. The final pH in glucose broth was determined by inoculating beef-infusion broth containing 1 per cent glucose and incubating anaerobically for four days. The pH was then determined directly by means of the glass electrode, thus avoiding the considerable error involved in the use of indicators. At least two tests were made on each culture and suitable controls of uninoculated medium and medium inoculated with cultures representative of low and high final pH[8] were included.

4. Growth in blood agar containing bile. Blood-bile plates were prepared by the addition of 5 per cent horse blood and 10 or 40 per cent ox bile respectively, to 3 per cent beef-heart agar. These plates were inoculated and incubated anaerobically for 48 hours at the end of which time they were inspected for growth.

[6] Lancefield strain 090R.
[7] Lancefield strain J17A4.
[8] Lancefield strains 090R and J17A4 respectively.

Control blood plates without bile were included in order to verify the viability of the organisms inoculated.

5. The fermentation of various sugars. The ability of the various strains of streptococci to ferment certain sugars was tested by growing the organisms in cooked meat medium to which 1 per cent of the sugar being tested had been added. Cultures were incubated for four days under vaseline seal, and the presence of acid was determined by the use of an indicator consisting of equal parts of 1.6 per cent alcoholic solution of brom-cresol purple and a 1 per cent alcoholic solution of cresol red, added directly to the culture. The carbohydrates used were glucose, lactose, maltose, inulin, mannitol and salicin. Controls of inoculated medium without carbohydrate were included.

B. Serological methods

1. Preparation of antisera. Antisera were prepared by a slight modification of the method of Lancefield (1933). The sedimented organisms from 500 ml. of anaerobic culture in cooked meat medium were collected and resuspended in $\frac{1}{100}$ volume of saline. The bacteria were killed by heating in the water bath at 60°C. for one hour and then stored in the refrigerator. Just before using, portions of the suspension were re-diluted twenty times with saline.

Rabbits were inoculated intravenously with 1 ml. of the diluted suspension daily for five days, and after a five day rest period, with 2 ml. daily for a similar length of time. Two additional courses of 4 ml. and 6 ml. of diluted suspension were given, and after a five to seven day interval, the rabbits were bled from the heart. The titer of the serum produced was tested by means of precipitin reactions against extracts of homologous organisms, and in most cases was found to be good. In a few cases, however, the titer was poor and a further course of injections was carried out daily for five days with the equivalent of 50 ml.[9] of living broth culture. The low virulence of the organisms was demon-

[9] 250 ml. of a forty-eight hour culture in cooked meat was centrifugated and the sediment resuspended in 30 ml. of saline. The animal was then given 6 ml. of this concentrated suspension for five doses.

strated by the ability of the animals to withstand such large doses.

After bleeding, which was carried out under strictly aseptic technic, the serum was stored in the refrigerator without the addition of any preservative, for earlier in the work the presence of formalin even in small concentrations (0.2 per cent) was found to interfere with the precipitin tests.

2. Preparation of extracts. In order to prepare extracts of the organisms to be studied for use in the precipitin tests, the bacteria were grown in 500 ml. of cooked meat medium under vaseline seal for 48 hours at 37°C. By means of a syphon, the broth was then removed, leaving the meat in the culture bottle. The broth was centrifuged and the sedimented organisms were resuspended in 9.5 ml. of saline and 0.5 ml. of N/1 hydrochloric acid (final concentration of acid N/20). This suspension was immersed in boiling water for ten minutes, then rapidly cooled under running water and centrifuged. The supernatant was clear and in most cases possessed a faint yellowish tinge. It is realized that extracts of this type contain a variety of substances and are apt to be devoid of certain other substances present in the whole organism.

Knowing nothing of the number or chemical composition of antigens present in the anaerobic streptococci, it was decided to use the Lancefield extracts while recognizing the fact that they would not necessarily react in a manner similar to that observed with the hemolytic streptococcus. We hoped then to evaluate the discrepancies and from them to determine more satisfactory methods. Indeed, our results definitely indicate that certain reacting substances are either masked or absent in the crude extract, for it will be shown that serum prepared against the whole organism reacted with crude extracts of organisms of heterologous strain in a different manner from that in which they reacted with extracts of homologous strain.

3. Technique of precipitin tests. As is usual in precipitin tests, the quantity of extract was varied while the quantity of serum was held constant. Three tubes were used for each complete test and contained 0.1 ml. of antiserum previously diluted 1:1. To the first tube was added 0.2 ml. undiluted extract; to the second

0.2 ml. of extract diluted one in four, and to the third 0.2 ml. of extract diluted one in sixteen. Thus, the actual extract content in the various tubes was 0.2 ml.; 0.05 ml. and 0.0125 ml. respectively. Extract and serum controls for each antigen and each serum were always included. The extract was added slowly so that it formed a distinct layer on the serum. The tubes were then placed in the water bath at 37°C. for thirty minutes, after which they were inspected for ring formation. The tubes were then shaken, incubated for two hours in the water bath, stored in the refrigerator overnight, and read finally the following morning. The positive reactions were recorded on an arbitrary scale of from one to four plus.

IV. RESULTS

A. Biochemical

The results of the biochemical tests are summarized in table 3. In our attempt to determine whether or not a correlation between the various biochemical tests existed, the 26 strains were listed according to their relative propensities for growth in 10 and 40 per cent bile. Three fairly clear-cut groups were then discernible; the first five strains grew in medium containing 10 and 40 per cent bile; the next twelve strains (if ± reactions are discarded) grew in 10 but not in 40 per cent, while the remaining nine strains (again disregarding ±) grew in neither. The growth in bile was chosen for purposes of arranging the strains because it was believed to give the most definite group differentiation. The results of the other biochemical tests performed were then listed under the various strains.

1. The fermentation of trehalose and sorbitol. The results of this biochemical test are plain; in no case was the medium fermented. It is worthwhile to note that the presence of fermentation with its concomitant acid production was determined not alone by indicators but also by the use of the more accurate glass electrode.

While, with the latter method, some differences in pH were noted, these were relatively inconsequential, for the range of pH with all strains was from 7.59 to 6.10 in the case of trehalose, in which positive controls read 5.00 and negative 6.90, and from

7.55 to 6.63 in the case of sorbitol, in which positive controls read 5.42 and negative 6.81. Further, the actual pH of the various cultures was almost identical whether the organisms were growing in trehalose or in sorbitol. It seems apparent, therefore,

TABLE 3

STRAIN	FINAL pH IN GLUCOSE	GROWTH IN BILE		FERMENTATION OF		HYDROLYSIS OF SOD. HIPPURATE	FERMENTATION OF					
		10 per cent	40 per cent	Trehalose	Sorbitol		Gluco.	Malto.	Lact.	Inul.	Man.	Sal.
Syl	4.90	+++	+++	−	−	+	A	A	A	A	−	−
Tam	4.95	++++	++++	−	−	−	A	A	(A)	(A)	−	−
Eff	6.90	++++	++++	−	−	+	A	−	−	A	−	A
Hub	6.22	+++	+	−	−	+	−	−	−	−	−	−
Lin	6.71	+++	+	−	−	+	−	−	−	−	−	A
Kas	7.01	++++	±	−	−	+	−	−	−	−	−	−
Gow	6.69	+++	±	−	−	+	−	−	−	−	−	−
Dau	6.89	+++	±	−	−	+	−	−	−	−	−	−
Mik	4.95	++	±	−	−	−	(A)	−	−	−	(−)	−
Mur	6.83	++	±	−	−	+	−	−	−	−	−	−
Syl II	6.96	++	±	−	−	+	A	−	−	−	−	A
Odo	6.25	++++	±	−	−	+	−	−	−	−	−	−
Hod	6.82	++++	±	−	−	+	A	−	−	−	−	−
Ase	7.10	++++	−	−	−	+	−	−	−	−	−	−
Rze	6.05	+++	−	−	−	+	A	−	−	−	−	−
Len	5.10	++	−	−	−	+	A	A	(−)	(−)	−	−
Fer		+++	−	−	−	+	−	A	−	−	−	A
Myt	7.19	±	−	−	−	−	−	−	−	−	−	−
Odu	6.10	±	−	−	−	+	−	A	−	−	−	−
Myi	5.60	±	−	−	−	+	A	A	A	−	−	A
TerA	6.69	−	−	−	−	+	−	−	−	−	−	−
TerB	6.36	−	−	−	−	+	−	−	−	−	−	−
Kud	7.16	−	−	−	−	−	−	−	−	−	−	−
Dre	7.20	−	−	−	−	−	−	−	−	−	−	−
Def	7.49	−	−	−	−	−	(−)	(−)	(−)	(−)	(−)	(−)
Cob	5.50	−	−	−	−	+	(−)	(−)	(−)	(−)	(−)	(−)

that since none of the anaerobic streptococci investigated ferment either of these sugars, their use is of no aid in classifying the strains. Furthermore, since the great majority of strains of hemolytic streptococci described by Lancefield (1933) ferment either one or both of the sugars used, another differential point

between anaerobic and hemolytic streptococci may be noted. In the lack of the ability to ferment trehalose and sorbitol, they most closely resemble *Streptococcus equi.*

2. The hydrolysis of sodium hippurate. Twenty of the twenty-six strains hydrolyzed sodium hippurate (see table 3). The grouping of the strains according to this characteristic, however, appears to bear no close correlation to the grouping on the basis of growth in bile. It may be noted, however, that four of the six negative strains occur in the group which fails to grow in media containing bile, while one occurs in each of the other groups. Put in another way, 80 per cent of the strains which grow in both 10 and 40 per cent bile also hydrolyze sodium hippurate; 92 per cent of strains which grow in 10 but not in 40 per cent bile, hydrolyze the hippurate, while only 55 per cent of strains which grow in neither 10 or 40 per cent bile exhibit the property of hydrolysis. It must be borne in mind, however, that chance, in all probability, plays a major rôle in determining this apparent correlation, since the series is too small for statistical analysis.

Probably, therefore, the only safe conclusion is that over 75 per cent of the strains of anaerobic streptococci investigated had the property of hydrolyzing sodium hippurate. In this respect they correspond most closely to Lancefield Group B (chiefly bovine strains) hemolytic streptococci.

3. Final pH in glucose broth. Reference to table 3 will disclose the final pH noted after four days' anaerobic growth in 1 per cent glucose broth, as determined by the glass electrode. It will be observed that only four strains produced a pH below 5.2 and only three strains were below 5.0. The remainder of the strains (twenty-one) varied from 5.5 to 7.49, the average pH produced being 6.65. The great majority of the strains, 84 per cent, would thus be included among the low acid producers which correspond to hemolytic streptococci of human origin (Edwards (1932), Lancefield (1933)). There is again, however, no correlation between the final pH and the grouping according to growth in bile, two of the four high acid producers falling in group 1, and two falling in group 2. Furthermore, only two of the strains produc-

ing a pH below 5.2 (presumably animal strains) fail to hydrolyze sodium hippurate, a characteristic noted in hemolytic streptococci of bovine origin by Edwards (1932) and others. Contrariwise, four strains producing low acidity (and thus presumably of human origin if the criteria applied to hemolytic streptococci hold) failed to hydrolyze sodium hippurate. If we take the groups as a whole, however, 18 of the 21 strains producing low acidity also hydrolyzed sodium hippurate (86 per cent).

4. The fermentation of various sugars. Of the 26 strains tested against the six sugars, fourteen failed to ferment any (see table 3). It is interesting to note that half of the strains which failed to ferment occur in the group of strains which fail to grow in 10 and 40 per cent bile. Six of the remaining seven non-fermenters occurred in the group which grows in 10 per cent but fails to grow in 40 per cent bile, while only one strain in the group which grows in both 10 and 40 per cent bile failed to ferment one or more of the test sugars employed. In other words, it appears possible that there is a positive correlation between the ability to grow in bile and the ability to ferment sugars. Further, of the three strains which ferment four sugars (Syl, Tam, Myi) two occur in the small (5) group of organisms growing in bile, while one occurs in the much larger (9) group which fails to grow in bile.

It appears that no strain thus far studied will ferment mannitol and that the remainder of the sugars are fermented in order of decreasing frequency as follows:

	strains
Glucose	9
Maltose	6
Salicin	5
Lactose	3
Inulin	3

The significance of the foregoing observations cannot, at the present time, be evaluated and it is highly probable that such correlations as appear to exist are wholly coincidental, especially in view of the fact that the series of strains is small in number.

B. Serological[10]

1. The results of the precipitin[11] tests performed as described under "Methods" are presented in table 4. It will be noted that sera against 24 strains of anaerobic streptococci were tested against extracts of the same 24 strains, and the results of the tests in each dilution of extract (undiluted, 1:4 and 1:16) were added and expressed as a number in the table—in order to approximate the titer quantitatively.

It will be seen at once that the organisms used were definitely antigenic and sera of fairly good titer were secured against 20 of the 24 strains. The remaining four strains (Mur, Len, Fer, Myi) while they produced a serum capable of reacting, gave poor results against the homologous extract. In some instances (Fer serum vs. Lin extract; Mur serum vs. Lin, Fer, Odu extracts) the serum gave better precipitin reactions against heterologous extracts. Undoubtedly, some of these apparent discrepancies may be explained on the basis of variation in both the extract and the serum due to storage over fairly long periods, since it was manifestly impossible to perform all the tests simultaneously. In addition, there may have been considerable variations in extract strength, since it was necessary to prepare more than one batch of each during the course of the work.

It must be remembered, in attempting to interpret the results presented in table 4, that the serum is prepared against the whole organism and that therefore it contains, theoretically at least, antibodies against each antigen present in the organism. The extracts contain a variety of substances both protein and carbohydrate in nature, since they are crudely prepared (see Methods).

[10] It should be noted here that attempts to perform agglutination tests were made but results from such tests were erratic, inconclusive and not reproducible, largely because of spontaneous agglutination. For that reason, results will not be given here.

[11] In order to eliminate the possibility that antibodies against constituents of the cooked meat medium might have been accidentally produced and thus might give false positive reactions with extracts of the organism, (although the latter extracts were made from carefully washed culture) extracts of cooked meat medium were made and tested against six antisera. The results were all negative.

It is in all probability true that there is a quantitative reduction in the amount of each antigen present, and it is also possible that there is a quantitative difference between antigens present in the whole organism and reacting substances present in the extract.

TABLE 4

Condensed results of precipitin reactions

EXTRACT	SERUM																						
	Syl	Tam	Eff	Hub	Lin	Kas	Gow	Dau	Mur	Syl II	Odo	Hod	Ase	Rze	Len	Fer	Myt	Odu	Myi	TerA	TerB	Kud	Dre	Mik
Syl	12	0	6	2	2	0	0	0	2	4	1	0	0	3	0	0	0	0	0	1	3	0	0	0
Tam	0	11	0	0	0	0	0	3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Eff	0	0	12	0	11	0	5	0	0	6	1	0	0	8	0	1	0	0	0	6	9	0	0	0
Hub	6	0	2	10	0	0	0	0	0	8	1	0	0	0	0	6	2	0	0	0	0	0	0	0
Lin	0	0	11	0	10	0	0	0	10	1	2	0	0	6	0	12	0	0	0	9	9	0	2	0
Kas	0	0	1	0	0	11	0	4	0	1	2	0	3	0	0	4	0	0	0	0	0	1	2	0
Gow	0	0	11	0	0	0	11	0	3	6	1	0	0	2	0	6	0	0	0	5	4	0	0	0
Dau	0	0	0	0	0	0	0	12	3	0	1	0	0	1	0	0	0	0	0	0	0	3	7	0
Mur	0	0	12	0	12	0	0	0	5	6	1	0	0	11	0	0	12	0	0	11	11	0	0	0
Syl II	0	0	3	0	2	0	0	0	0	8	2	0	0	1	0	0	0	0	0	2	2	0	3	0
Odo	0	3	0	0	1	0	5	1	0	0	11	0	0	1	0	6	0	0	0	7	6	3	0	0
Hod	0	0	0	0	0	0	0	0	0	0	4	11	6	0	0	0	0	0	0	0	0	1	4	0
Ase	0	0	0	0	0	0	0	0	0	0	4	0	10	0	0	0	0	0	0	0	0	2	1	0
Rze	0	0	9	0	10	0	2	0	0	1	0	0	0	8	0	0	0	0	0	4	10	0	0	0
Len	0	0	0	0	0	0	0	0	0	0	0	0	0	0	7	0	0	0	0	0	0	0	2	0
Fer	0	0	9	0	0	0	3	0	9	0	0	0	0	0	0	4	3	0	0	6	8	0	0	0
Myt	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	6	11	0	0	2	0	0	0	0
Odu	0	0	2	0	10	0	0	0	8	6	0	0	0	4	0	0	0	10	0	1	3	1	2	6
Myi	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	4	1	0	0	0	0
TerA	0	1	10	0	5	0	0	0	0	2	2	0	0	0	0	0	0	0	0	10	12	0	1	0
TerB	0	2	11	0	8	0	0	0	0	5	8	0	0	2	0	0	0	0	0	11	12	0	2	0
Kud	0	0	0	0	0	0	0	0	0	0	5	0	0	0	0	0	0	0	0	2	0	12	11	0
Dre	0	0	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	10	12	0
Mik	0	0	2	0	0	0	0	0	1	0	3	0	0	0	0	3	2	0	0	1	0	3	2	11

With these facts in mind, cross-reactivity in extract-serum mixtures does not necessarily imply that there are similar reacting substances in each extract, but may simply indicate that the serum contains antibodies against two or more substances, one of which is present in one extract and another in the second, and both of which must have been present and antigenic in the whole organism used for producing the immune serum. This possibility

presents a serious barrier to the grouping of these strains on the basis of the possession of a similar reacting substance in the extracts, at least until extracts can be prepared containing only the antigen desired. As an illustration, it will be noted that serum Fer reacts, among others, with extracts Gow, Myt and Eff. Likewise, Sera Gow, Myt and Eff react with extract Fer. Despite this cross-reactivity, however, if we assume that the reaction is on the basis of the possession of one common reactive substance, we are at a loss to explain the failure of the serum Myt to react with extract Gow, since each organism must have possessed an antigen in common with an antibody in serum Fer, in order to react with serum Fer. Consequently, it must be assumed that the reactions are *not* on the basis of *one* common antigen, but rather depend on the possession of multiple antigens. If this be true, then the Fer-Gow reaction might very conceivably be predicated on a common antigen "A" and the Fer-Myt reaction on a common antigen "B". Thus Myt and Gow, while both reacting with serum Fer, would not necessarily react with each other. There are many examples of this type of apparently anomalous cross-reaction throughout the series and their occurrence renders practically impossible the serological classification of the anaerobic streptococci by means of simple precipitin tests.

A further stumbling block is noted in the fact that some sera (Kas, Hod, Odu, Len, Myi) react with none but their homologous extracts while these extracts react with several heterologous sera. In the cases of Len and Myi, the cross-reactivity of the extracts is limited, and since the titer of the homologous sera has been shown to be poor, it is conceivable and even probable that with better sera, similar cross-reactions would be observed. However, in the cases of Kas, Hod and Odu, cross-reactions were observed against 8, 4 and 9 sera respectively, and since the sera against these organisms were of good titer, it is extremely difficult to explain the apparently paradoxical results.

Table 5 presents in a simpler form the cross-reactive extracts noted under each serum, together with an indication of the extracts which check on reversal and those which do not.

From a consideration of the results, it becomes increasingly

TABLE 5

SERUM																							
Fer	Gow	Dau	Myt	Odu	Lin	Odo	Syl II	Tam	Hod	Rse	Myi	Ase	Kas	Kud	Len	Hub	Mur	Syl	Eff	Dre	TerA	TerB	Mik
Extracts giving reciprocal cross reactions																							
Gow Myt Eff	Fer Eff		Fer		Rse Mur Eff TerA TerB	TerA TerB Kud	Eff TerA TerB			Lin Eff TerB				Odo Dre		Syl	Lin	Hub	Fer Gow Rse Lin TerA TerB Syl II	Kud	Odo Lin Eff TerB Syl II	Odo Lin Eff TerA Syl II Rse	
Extracts giving cross reactions only																							
Kas Odo Hub Lin Mik	Odo	Kas Tam	Mik Hub Mur		Syl Odu Syl II Kud	Kas Mik Syl Lin Hod Ase Syl II	Gow Hub Syl Mur Odu	Odo TerA TerB	Dau	Gow Syl Mur Odu		Kas Hod		Dau Mik Ase			Fer Dau Gow Syl Odu		Hub Syl Mur Odu	Dau Kas Mik Lin Hod TerB Odu Syl II Len	Fer Gow Mur Kud Rse Myt	Fer Gow Mur Syl Odu	Odu
Total cross-reacting																							
8	3	2	4	0	9	10	8	3	1	7	0	2	0	5	0	1	6	1	11	10	11	11	1

evident that the serological classification of strains of anaerobic streptococci cannot be accomplished by the methods which we have used. It is seen that in the first place there is a great diversity of groups, and secondly, that the boundaries of these groups are not clear-cut. Perhaps our work supports the findings of Andrewes and Christie (1932) with regard to hemolytic streptococci. These workers examined streptococci from a variety of sources and came to the conclusion that "only exceptionally are two strains of streptococci serologically identical and very rarely are they entirely dissimilar. Almost always there is more or less *group antigen* in common between them; there are definite hints that more than one group antigen exists."

TABLE 6

SERUM	EXTRACTS																							
	Syl	Tam	Eff	Hub	Lin	Kas	Gow	Dau	Mur	Syl II	Odo	Hod	Ase	Rze	Len	Fer	Myt	Odu	Myi	TerA	TerB	Kud	Dre	Mik
J17A4	0	0	0	0	0	0	0	0	1	0	0	0	0	0	1	0	0	0	6	0	0	0	0	0
O90R	0	1	0	0	1	0	0	0	1	0	0	0	0	0	1	0	0	0	0	0	0	0	6	0
K104	0	0	0	0	0	8	1	7	0	0	0	0	0	0	0	2	0	1	0	1	1	9	0	0

Further consideration of serological procedures which may be of use in the future will be given in "Discussion."

2. The relationship between anaerobic streptococci and hemolytic streptococci (Lancefield groups A, B and C) on the basis of serological findings. By testing sera prepared against hemolytic streptococci of groups A, B and C, according to the method of Lancefield, against extracts of the 24 strains of anaerobic streptococci, some definite cross-reactions were noted. The hemolytic strains[12] used as antigens were kindly supplied by Dr. Lancefield of the Rockefeller Institute. The results are indicated in table 6 and are again expressed as the sum of the results in three dilutions. Control tests of each of the three sera against their homologous extracts were all strongly positive (12) and against the two heterologous extracts were all negative.

[12] Strain J17A4, Group A. Strain 090R, Group B. Strain K104, Group C.

In all, 14 of the 24 strains of anaerobic streptococci cross-react to some extent at least with anti-hemolytic streptococcus serum of groups A, B or C. Numerically, by far the greatest cross-reactivity occurs in group C, where 8 strains are found to react, 3 of them strongly; 5 strains cross-react in group B, only one strongly; 3 strains in group A, one strongly. Two of the 3 strains which cross-react in group A also cross-react in group B. The strains cross-reacting in group C apparently do not cross-react in either group A or B.

Again we can conclude nothing definite from these reactions, except that it is probable that at least one and probably more than one antigenic substance present in the hemolytic streptococcus is also present in some strains of anaerobic streptococci. Further, it is possible that the group cross-reactivity noted may be utilized in a serological classification of the anaerobic streptococci. Such a procedure, however, would be entirely arbitrary as yet, since there is no apparent correlation between the grouping in this manner and the possible grouping based on any one of the other biochemical or serological procedures so far performed.

V. DISCUSSION

The attempt to classify the anaerobic streptococci by means of their cultural characteristics, biochemical properties, or immunological reactions, is one which is beset with many difficulties. True, grouping by the adoption of arbitrary criteria in any one of these fields is possible, but it is found immediately that such grouping is of no value insofar as the study of the organism is concerned, since overlapping of the groups with respect to other criteria invariably occurs. For example, there is no apparent value in cultural classifications either on the criteria we have used (see tables 1 and 2) or in those proposed by Colebrook and Hare (1933) or Prevot (1925), since organisms in one group in such classifications are scattered widely when classified according to biochemical methods (e.g., growth in bile or final pH) and still further scattered when compared by means of their immunological properties. Further, no one has shown that such a

cultural classification is of value in determining pathogenicity. Similar objections attend the use of a strictly biochemical classification and here, indeed, it has been demonstrated that there is no correlation between any two reactions investigated, much less between cultural or immunological properties.

The fact that classifications of other organisms based on cultural or biochemical characteristics have in the main been shown to be purely arbitrary and have been abandoned in favor of the much more satisfactory serological methods, leads one to the belief that future work along serological lines holds the greatest promise. The preliminary serological studies reported above, however, leave much to be desired. They fail entirely to provide a clear-cut grouping of the organisms and, in addition, show many apparently paradoxical reactions. Such reactions seem to depend for their explanation upon the assumption that each organism contains not one but several antigenic fractions, certain of which differ in different members of the group studied. For this reason it seems logical to attempt the solution of the problem by fractionation of the soluble portions of the various organisms with the eventual hope that the cross-reactive substances may be identified more exactly and that a classification based on the presence or absence of definite antigenic substances may be evolved in a manner similar to that which exists for hemolytic streptococci. Such work is at present being undertaken, and early experiments seem to indicate that at least two antigens play a part in the precipitin reaction. One of these is precipitated from suspensions of lyophilized and ground organisms by HCl at a pH of 3.5, is Biuret and Molisch positive and has an N:P ratio of from 3 to 4. As shown by precipitin tests, it does not react specifically with homologous serum but cross-reacts with several of the sera tested. Another fraction, soluble in HCl at pH 3.5 but precipitated by 50 per cent trichloracetic acid in the proportion of 0.15 to 0.20 ml. per milliliter of solution, shows an N:P ratio of about 1.5 and is Molisch positive and Biuret negative. Thus far this fraction reacts only with homologous antiserum. It should be remembered, however, that the above descriptions are tentative in that the work done has been

limited and thus far has been carried out on only four strains. Further, it will probably be necessary to determine the antigenicity of such fractions and to prepare antisera against them rather than to utilize antisera against the whole organisms as we have done.

Attempts to prove pathogenicity in laboratory animals have thus far failed completely in mice, rabbits, rats, guinea-pigs and monkeys, although only a few strains were utilized. In no case could death of an animal be attributed to the organism, but in one guinea-pig a small indolent abscess was found. In the few experiments which were attempted, the addition of mucin or the preliminary injection of mucin was without effect. This apparent lack of pathogenicity for laboratory animals has been noted by many other observers, despite the fact that clinically there is little doubt that anaerobic streptococci are productive of severe and frequently fatal infections under certain conditions. Further work on this problem is at present going forward.

VI. SUMMARY

The results of the study of 26 strains of anaerobic streptococci along biochemical and immunological lines are presented. These strains were isolated from parturient and post-abortal women. No correlation between any two biochemical methods of grouping could be discerned, nor was it possible to form definite groups of these strains by means of the precipitin reactions. Nevertheless, it is undoubtedly true that certain antigens are present in common and the study of such antigens may well aid in classifying the organisms.

It is well to bear in mind that most of the strains of organisms which we investigated might not have had any etiological relationship to the infection that existed at the time they were isolated and, indeed, in many cases, they were known to have had no rôle in the infection, probably existing only as harmless saprophytes. Only one strain (Ter B) isolated from the blood stream can unquestionably be regarded as pathogenic. It is therefore entirely possible that when more definitely pathogenic strains (i.e., strains from the blood stream) are studied, the results may be entirely different and more coherent.

VII. CONCLUSIONS

A study of the biochemical reactions in 26 strains of anaerobic streptococci justifies the following conclusions:

1. Certain strains grow in both 10 and 40 per cent bile, some grow in 10 and not in 40 per cent bile, and a third group will not grow in media containing 10 per cent or more of bile.
2. On the whole, the anaerobic streptococci produce little acid from glucose broth, the final pH being above 6.0 in over 75 per cent of the strains studied.
3. No strain ferments either trehalose or sorbitol.
4. Over 75 per cent of the strains hydrolyze sodium hippurate.

From a study of the precipitin reactions, the following conclusions may be drawn:

1. Whole heat-killed anaerobic streptococci are antigenic in rabbits, producing precipitins which are not entirely specific.
2. Since the organisms cross-reacting in precipitin tests are not identical as shown by other tests, the cross-reactions must be explained on the basis of antigens in common.
3. From examination of the results, it is necessary to conclude that the anaerobic streptococci contain at least two and probably more than two different antigenic substances.
4. There is some evidence that a relationship exists between certain anaerobic streptococci and groups A, B and C hemolytic streptococci.
5. It is impossible as yet to formulate clearly a classification of the anaerobic streptococci by means of serological methods. The evolution of such a classification will probably depend ultimately on the successful fractionation of the components of the organisms and the production of satisfactory antisera against each fraction.

In preliminary experiments, the anaerobic streptococcus has not been found to be pathogenic for the usual laboratory animals.

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