THE CONSTANCY OF THE CULTURAL REACTIONS OF THE STREPTOCOCCI ON HOLMAN'S CARBOHYDRATE AND BLOOD AGAR MEDIA.

- - - -

A CONTRIBUTION TO THE CLASSIFICATION OF STREPTOCOCCI.

PREFACE

Before entering into a more detailed account of the results of this research, it might be fitting to give a short account of the amount of personal care and work used in these observations.

The preparing, adjusting and sterilization of all media, the sterilization of all apparatus used throughout these experiments was carried out personally. The blood for the blood agar media I withdrew from patients under treatment for syphilis; the sheep's blood for use as serum in the sugar media I obtained direct from the cattle markets and, on separation of the clot, diluted and filtered the serum.

The isolation of all cultures, the inoculation of all media, the investigation of all films of the sugar test cultures for purity and presence of growth, were all personal work. In the absence of a vivisection license, the testing of the non-haemolytic strains of streptococci for pathogenicity was very kindly carried out by the Glasgow Public Health Laboratories.

And then in the first place the work was made possible by the grant of a Muirhead Scholarship, and

by permission to work in the Victoria Infirmary Laboratory under the kindly direction of Dr. John Anderson, to whom and to the Muirhead trustees I herewith express thanks.

August 1930.

THE CONSTANCY OF THE CULTURAL REACTIONS OF THE STREPTOCOCCI ON HOLMAN'S CARBOHYDRATE AND BLOOD AGAR MEDIA.

A CONTRIBUTION TO THE CLASSIFICATION OF STREPTOCOCCI.

The presence of streptococci in air, earth and water, their presence in the whole length of our alimentary canal, the presence in mucous membranes of apparently normal individuals of streptococci, with difficulty distinguishable from those causing the most acute cellulitis, or grave endocarditis, the technical difficulties and differences of opinion found in the study of these organisms, have made them a subject of undoubted interest and importance during the last thirty years.

Koch first described the streptococcus in 1878, but it was the beginning of the twentieth century before general interest in the species was aroused in this country. In 1901 Libman (1) described the streptococcus as with other bacteria being producers of acid when grown in serum or sugar media, and while Marmorek first noted that streptococci had haemolytic power it was Schottmuller in 1903 who by their action in blood agar differentiated three varieties, those which produce haemolysis, those which produce green colouration and those which produce

colonies of a slimy consistence. In 1905 Gordon (2) set about classifying streptococci by their action on carbohydrate media and advocated the use of his nine tests as a definite basis of classification.

GORDON'S TESTS

1. The question of clotting of litmus milk in three days at 37°C.
2. The reduction of neutral red broth during incubation, anaerobically, for two days at 37°C.
3. The production of an acid reaction aerobically, in three days at 37°C. when cultivated in slightly alkaline broth containing 1% of saccharose.

Tests 4, 5, 6, 7, 8, 9, are exactly similar to test 3, except in so far as 1% lactose, 1% raffinose, 1% inulin, 1% salicin, 1% coniferin, 1% mannite respectively, are introduced in place of 1% saccharose.

At this period the morphology of the streptococcus was extensively studied by most workers and found to be of very slight value as a means of differentiation; and pathogenicity of the same strain was found to vary so much when subcultured in vitro, and when exalted by animal passage, as to be a doubtful criterion of differentiation (31).

New methods of differentiating streptococci by growth appearances on particular blood agar media have been recently described by Crowe (3) and by Smith and Brown (4)

but these methods did not find general acceptance. Since 1914 serological tests have been extensively studied especially as a means of classifying and identifying specific haemolytic strains. While these researches have been productive of tests of presumptive value only in the recognition of haemolytic strains, there have emerged sera of marked therapeutic value in the treatment of scarlet fever and to a lesser extent in the treatment of allied diseases of supposed streptococcal origin. In 1921 a notable advance in the classification of streptococci of faecal origin was made, when J.H. Dible (5) described the value of the heat test in differentiating the enterococcus.

But since 1905 the bulk of the literature on streptococcal classification has dealt with the fermentation by streptococci of carbohydrate media. Using Gordon's nine tests and taking into account morphology and pathogenicity, Andrewes and Horder (6) made an extensive study of streptococci pathogenic for man. By these tests they declared they were able to identify and classify streptococcal strains. Naturally variations and modifications of Gordon's tests were tried. Many subsequent observers reduced the number of test substances. For some time the amount of acid formed in the

carbohydrate media was titrated as affording a more accurate means of differentiating between strains. This procedure advocated by C.E.A. Winslow (7) prevailed chiefly in America. In 1913 Broadhurst (8) showed that the amount of titratable acid varied directly with the suitability of the medium for vigorous growth. In 1919 Avery and Cullen (9) found that the limiting hydrogen ion concentration of a streptococcal dextrose culture was remarkably constant for any particular strain of streptococcus, and that there was a distinct difference in the limiting hydrogen ion concentration of human and bovine haemolytic strains. Though found to be a satisfactory presumptive test by Avery and Cullen, J. Howard Brown (11) reports that the limiting hydrogen ion concentration is not of itself of service in differentiating strains and results of the method have not been extensively reported.

Gordon (30) and Andrewes and Horder (29), these latter admitting that "the constancy of these reactions is clearly a cardinal point" (12), were each satisfied that the qualitative fermentation reactions were sufficiently constant to afford a basis of classification. Floyd and Wolbach (13), Lyall (14), Holman (15) and Blake (16) after extensive studies of these organisms were also satisfied that these reactions were constant and of

value, and yet there creep into the literature from time to time reports of the inconstancy or inadequacy of these reactions.

Ritchie (17) states "while admitting that the number of experiments described is far too small and the time over which they have been extended is far too short to permit of my dogmatizing as to the possible connection between prolonged subculture and biochemical activity, I venture to suggest they indicate a possibility of error in the classification of any given strain of streptococcus, on the strength of a single examination of its carbohydrate reactions."

D.H. Bergey (18) "Unfortunately the streptococci are very easily affected in their fermentative powers, so that it is not an easy matter to obtain concordant results upon repeating the culture tests after the organisms have been grown in artificial media for some time."

David John Davis (19) "Sugar fermentation with the streptococcus group has on the whole not been trustworthy or satisfactory on account of the inconstancy of results."

Kliger (20) "That the different cells of the same strain may vary somewhat in their power to ferment a certain sugar is an established fact."

Beattie and Yates (21) state that "Gordon's tests have

proved quite unreliable for differentiating strains" though they also state with what few strains they tested for constancy "that the reactions have been fairly constant" (27).

In 1911 E.W.A. Walker (22) stated "It has been observed that when observed over extended periods of time or after changes in their environment likely to encourage the appearance of variations, these reactions of the streptococci exhibit an extraordinary degree of variability."

In 1924 Crowe stated (23) "Can we rely on a streptococcus culture remaining constant after repeated subculture? The matter is in doubt."

In these statements, there is some evidence, that the constancy of the cultural reactions of the streptococci is still unsettled.

The Inconstancy of the Haemolytic Tests.

Though Marmorek had noted the haemolytic acitivity of streptococci, and Schottmuller in 1903 had differentiated streptococci by their haemolytic power, there was no attempt to combine haemolytic power with carbohydrate fermentation tests, as a means of classification, until 1914. Till then the stability of haemolytic power under cultural conditions had not been much investigated, and thereafter, though perhaps investigated, the stability of

the haemolytic power did not give rise to discussion.

Floyd and Wolbach (25) in 1914, state "While most of the organisms have retained their haemolytic power for several weeks, a number of cultures lost this property after several months cultivation on blood serum .. .. .. In no instance did the cultures which were non-haemolytic acquire the property of haemolysis while under cultivation." No details of the experiments are given.

E. C. Rosenow (24) 1914 describes a case in which "the transformation of a haemolysing streptococcus to a green-producing streptococcus occurred on blood agar with peptone and beef extract, the supply of oxygen being abundant." In this experiment a haemolysing strain, after being twice plated out from a single colony, and kept by occasional subculture for fifteen months, was sewn on a peptone and blood agar medium, with peptone and beef extract, as stated, and left in "uncorked tubes" for one month. At the end of which time, sub-cultures shewed both haemolytic and non-haemolytic colonies. He describes, in the same article how, by various other means, (E.G. symbiosis) twenty other haemolytic strains became non-haemolytic. There is also a description of how several strains of streptococcus viridans, by various means, (E.G. high oxygen tension) became haemolytic streptococci.

In 1919 McLeod (26) described streptococci which lost their haemolytic power one in one month, and one in three months after isolation.

There have, however, mostly been reports of the inconstancy of haemolytic or non-haemolytic power as of an exceptional occurrence.

In 1920 B. J. Clawson (37) states "Haemolysis, as shewn in this series of 134 strains, is constant after nearly two years of artificial cultivation."

The uncertainty to which such opposing views, both as regards the carbohydrate fermentation and, to a lesser extent, the haemolysis tests gives rise, the growing tendency since 1914 to combine carbohydrate tests with tests for haemolytic power as a means of classification, the simple classification proposed by W. L. Holman accepting the constancy of both these reactions, determined the subject of this research - the observation over a period of nine months of eighteen strains of streptococci on Holman's carbohydrate and blood agar media.

HOLMAN'S BLOOD AGAR MEDIA.
(Extract. Journ. Med. Research Vol. 34 1916 p. 381.)

Blood agar: Plain agar (.6 plus) is sterilized in one hundred cubic centimeter quantities in flasks. A flask is heated in the autoclave and placed in the paraffin oven at 58°C for some time. It is cooled to 50°C and

five cubic centimeters of defibrinated human blood is thoroughly mixed with the fluid agar. This mixture is poured into petri dishes for blood agar plates, and on the surface of previously prepared agar for blood agar slants, to a depth of about two millimeters. The basic agar for these slants is made of 1.5 per cent agar in normal saline (.85 per cent sodium chloride) filtered tubed, sterilized and slanted. This agar base is clear and colourless and makes the whole surface of the finally prepared blood agar slant available for the study of the growth of the bacteria.

HOLMAN'S CARBOHYDRATE MEDIA
(Extract Journ. Med. Research Vol. 34 1916 p.385)

Take two hundred cubic centimeters of double strength broth 1.2 plus, add to this one hundred cubic centimeters of water, four grammes of the test substance and four cubic centimeters of Andrade's indicator (decolourized acid fuchsin.) This is sterilized in a large flask on three successive days in flowing steam. Beef serum diluted one-half with water is slowly filtered through a Berkefeld filter, and two hundred cubic centimeters added to the above. The whole is then tubed through a sterile funnel into sterile test tubes, and the tubes incubated two days to eliminate chance contaminations.

The factors of such an investigation are the test media, the strains of streptococci, and the routine followed in the investigation.

Test Media: The test media employed in this investigation were blood agar, and lactose mannite and salicin serum broths - the media by the reaction to which streptococci are identified in Holman's classification (32).

The blood agar (33) prepared in bulk, consisted of nutrient agar to which was added 5% of defibrinated human blood. Except in adjusting the media to pH 7.8, the nutrient agar was prepared as described, and the blood added at between 45°C. to 50°C. The blood plates were incubated at least 24 hours to ensure their sterility before inoculation.

In the preparation of the carbohydrate media, double strength broth, Andrade's indicator (35) sugars and serum with slight variations were used in the quantities and methods described. The double strength broth was adjusted to pH 7.8, and the whole sterilised. "Difco" brand sugars were used and added to the broth as 10% solutions in distilled water after being sterilised for one half hour at 100°C. The sugar broths were thus sterilised in the Koch only 15 minutes on three successive days. Instead of beef serum an equal volume

of sheep serum was diluted, filtered and added to the sugar media as described.

The media were incubated two days to ensure sterility. Several tubes of the sheep serum broths without the addition of the sugar were inoculated, some with B.Coli and some with strains of streptococci. No acid production was obtained and the media were found reliable.

Strains of Streptococci Used: For this research eighteen strains of streptococci have been employed - eight strains of haemolytic streptococci and ten strains of non-haemolytic streptococci. These strains were taken from the routine laboratory sources, and are not representative of the various classified types of streptococci. Among the haemolytic group there are five strains of streptococcus pyogenes, one strain of streptococcus anginosus, and two streptococcus equi. The high percentage of streptococcus pyogenes is in accordance with their frequency - (762 strains of streptococcus pyogenes being present in Holman's series of 1224 haemolytic strains). In the ten non-haemolytic strains there are present only streptococcus mitis and streptococcus salivarius (which between them represent more than 50% of Holman's non-haemolytic series.)

Having in mind the article on "Errors in Differentiation

of Streptococci" by Holman (34) these strains were each isolated from a single colony on blood agar, sown on broth, replated on blood agar and re-isolated from the same media on six occasions before any culture was accepted from which the stock cultures were obtained. The stock cultures were maintained in nutrient agar stabs and strains were occasionally re-isolated from single colonies in a search for variants.

In Holman's classification to exclude strains of pneumococci as far as possible all strains are examined for capsules and solubility in bile. Strains classified are non-capsulated and bile insoluble. In this series haemolytic power is accepted as excluding pneumococci and (a) all non-haemolytic strains which fermented Inulin serum water were rejected. (b) Of the ten non-haemolytic strains accepted eight survived cultivation in plain agar at three week intervals for six months. (c) at the end of that period seven of these were injected into mice using .5 cc. of a 24 hour broth culture. None were pathogenic. On these three facts it was concluded that pneumococci had been excluded.

The results recorded of the action of the strains on blood agar were observed, after 24 hours' incubation, on plates whose surface was smeared from one or two

loopsful of broth culture. The test sugar media were inoculated during the earlier tests by three loopsful of a 24 hours' broth culture - since streptococci are reported as growing more vigorously on fluid media - but latterly more frequent and vigorous growths were obtained by inoculating from a 24 hours' growth on blood agar. The test cultures were incubated at least seven days before lack of fermentation was recorded and to confirm all results films were examined from each culture tube and latterly also from agar slopes subcultured from each culture tube. Contaminated tubes, and tubes in which no vigorous growth had taken place were thus recognised.

Haemolytic.

Strain	Source	Name.	24 hours' broth Length of Chain	Pathogenicity	Inulin
I_3	From the spleen post mortem	Strept.pyogenes	Chains of 5-16 cocci	not examined	-
I_4	in a case of nephritis with purpura	Strept.pyogenes	"		-
K_2	From a superficial	Strept.equi	(Chains of 10-30	"	Neg.
K_3	skin lesion	Strept.equi	(Cocci conglomerate		Neg.
Q6	From the throat in a	Strept.pyogenes	8-10-30 cocci conglomerate	"	Neg.
Q20	case of subacute	Strept.pyogenes	8-10-15 cocci per chain	"	Neg.
Q60	rheumatic fever	Strept.pyogenes	8-10-16 cocci per chain	"	Neg.
N_3	From inflamed fauces in a case of exophthalmic goitre.	Strept. anginosus	Very long chains crossing stage	"	Pos.
		Non haemolytic.			
L2	From the faeces in a	Strept.mitis	Chains of 2-6 cocci but chiefly diplococci	Non-pathogenic.	Neg.
L3	case of tuberculous peritonitis	Strept.mitis or faecalis	Chains of 2-6 cocci but chiefly diplococci	"	Neg.
N1	From inflamed fauces	Strept.mitis	10-20 cocci per chain	"	(Pos. (Neg.
N2	in a case of	Strept.mitis or salivarius	Chains of 2,3,9 and 20 cocci	"	Neg.
N5	exophthalmic goitre	Strept. salivarius	Chains of 7 cocci	"	Neg.
N7		Strept.mitis or salivarius	Chains of 2-6 cocci	"	Neg.
N3(b)	From a contaminated blood plate	Strept.mitis	8-15 cocci per chain	"	Neg.
M4	From the throat in a	Strept.mitis		"	Neg.
M6	Case of sub-acute rheumatism	Strept.mitis or salivarius	30-50 cocci per chain and chains crossing stage	"	Neg.
Q11	From the throat in a case of sub-acute rheumatic fever.	Strept. salivarius or mitis	Chains of 6-11 cocci conglomerated: also diplococci	"	Neg.

I_3

Date	Action on Blood Agar.	Action on Lactose	Mannite	Salicin
27. 7.'26	Haemolysis	+	-	+
23. 8.'26	"	+	-	+
12.10.'26	"	+	-	+
12. 1.'27	"	+	-	+
16. 2.'27	"	+	-	+
20. 4.'27	Haemolysis			

The haemolytic strain I_3 has remained haemolytic after nine months' cultivation.

During seven months it remained constant in its carbohydrate fermentation reactions.

I_4

Date	Action on Blood Agar.	Action on Lactose	Mannite	Salicin
19. 7.'26	Haemolysis	+		+
14. 1.'27		+		+
9. 2.'27		+	-	+
28. 3.'27		+	-	+
20. 4.'27	Haemolysis			

The haemolytic strain I_4 has remained haemolytic after nine months' cultivation.

Strain I_4 has remained constant in its action

on lactose and salicin during eight months. The negative action on mannite during the first two tests is not recorded as the growth if present was poor.

K2.

Date	Action on Blood Agar.	Action on Lactose	Mannite	Salicin
20. 7.'26	Haemolysis	- -	-	+
24. 8.'26		-	-	+
19.10.'26		-	-	+
14. 1.'27		-	-	+
8. 2.'27		-	-	+
20. 4.'27	Haemolysis			

The haemolytic strain K_2 has remained haemolytic after nine months' cultivation.

The reactions on carbohydrates have not varied during seven months.

K3.

Date	Action on Blood Agar.	Action on Lactose	Mannite	Salicin
24. 7.'26	Haemolysis	-	-	+
21. 8.'26		-	-	+
14. 1.'27		(+	-	-
		⁂ (		
21. 1.'27		(-	-	+
8. 2.'27		-	-	+
20. 4.'27	Haemolysis			

⁂ (? tubes confused)

The haemolytic strain K3 has remained haemolytic in its action on blood agar after nine months' cultivation.

Strain K3 has remained constant in its reaction on carbohydrates but the test performed on 14th Jan. As on that date the tube showed evidence of growth and no contamination, the tests were immediately repeated and the original result obtained.

Q6.

Date	Action on Blood Agar.	Action on Lactose	Mannite	Salicin
17. 9.'26	Haemolysis	———		
21. 1.'27		+	-	+
12. 2.'27		+	-	+
13. 3.'27		+	-	+
20. 4.'27	Haemolysis			

The haemolytic action of Q6 has been retained after seven months' in culture.

The strain Q6 has remained constant in its fermentation reactions during two months. During the test of 13th March the fermentation of salicin did not appear for eleven days. A duplicate tube showed acid within seven days.

Q20.

Date	Action on Blood Agar.	Lactose	Mannite	Salicin
17. 9.'26	Haemolysis	———		
21. 1.'27		+	-	+
12. 2.'27		+	-	+
13. 3.'27		+	-	+
20. 4.'27	Haemolysis			

The haemolytic strain Q20 has remained haemolytic after a period of seven months in culture.

Strain Q20 has remained constant in its fermentation reaction on carbohydrates during two months.

Q60.

Date	Action on Blood Agar.	Lactose	Mannite	Salicin
17. 9.'26	Haemolysis	———		
21. 1.'27		+	-	+
12. 2.'27		+	-	+ *
13. 3.'27		+	-	+
20. 4.'27	Haemolysis	———		

*Acid appeared after 11 days.

Strain Q60 has retained its haemolytic action on blood agar after a period of seven months.

The carbohydrate fermentation reactions have remained constant during two months.

N3.

Date	Action on Blood Agar.	Action on Lactose	Mannite	Salicin
7. 8.'26	Haemolysis	+	-	-
22. 9.'26	Haemolysis	+	-	-

The haemolytic strain N3 remained haemolytic after six weeks on media.

The action on sugars has remained unaltered after six weeks' cultivation.

L2.

Date	Action on Blood Agar.	Action on Lactose	Mannite	Salicin
19. 7.'26	Non-haemolytic	+	-	+
12.10.'26		+	-	+
12.12.'26		+	-	
21. 1.'27		+	-	+

The non-haemolytic strain L2 which was retested as L2(a) and L2(b) has remained non-haemolytic over a period of nine months.

Strain L2 has remained constant in its fermentative reactions during six months.

In February 1927 strain L2 was re-sown on blood agar and two strains L2(a) and L2(b) isolated and tested. Strains L2(a) and L2(b) gave the reactions of strain L2.

L2 (a)

Date	Action on Blood Agar.	Action on Lactose	Mannite	Salicin
22. 2.'27	Non-haemolytic	+	-	+

L2 (b)

Date	Action on Blood Agar.	Action on Lactose	Mannite	Salicin
22. 2.'27	Non-haemolytic	+	-	+

N5.

Date	Action on Blood Agar.	Action on Lactose	Mannite	Salicin
9. 8.'26	Non-haemolytic	+	-	-
23. 8.'26		+	-	-
23. 9.'26		+	-	-

The non-haemolytic strain N5 died at the end of six weeks. The three groups of fermentation tests performed during that period gave identical results.

M4.

Date	Action on Blood Agar.	Action on Lactose	Mannite	Salicin
9. 8.'26	Non-haemolytic	+	-	+
14. 9.'26		+	-	+

The non-haemolytic strain M4 died after five weeks. The two groups of tests performed during that

period gave identical results.

N3 (b)

Date	Action on Blood Agar.	Action on Lactose	Mannite	Salicin
23.11.'26	Non-haemolytic	+	-	+
2. 2.'27		+	-	+
8. 3.'27		+	-	+
20. 4.'27	Non-haemolytic			

In N3 (b) the non-haemolytic action on blood agar has been retained during five months.

During four months' observation N3 (b) has shown identical reactions to sugars at each test.

L3

Date	Action on Blood Agar.	Action on Lactose	Mannite	Salicin
19. 7.'26	Non-haemolytic	+	-	+
Date lost		+	-	+
——		+	+	+
9.11.'26		+	+	+
14. 1.'27	Non-haemolytic	+	+	+

L3 (b)

Date	Action on Blood Agar.	Action on Lactose	Mannite	Salicin
8. 9.'26	Non-haemolytic	+	+	+
19.10.'26		+	+	+

The non-haemolytic strain L3 has remained non-haemolytic after a period of six months.

During the first two months' strain L3 remained constant. On subculture on blood agar strain L3 (b) was isolated. In L3 (b), and later in L3, the power to ferment mannite developed and remained.

Q11.

Date	Action on Blood Agar.	Lactose	Mannite	Salicin
14. 9.'26	Non-haemolytic	+	-	+
30.12.'26		+	-	-
2.'27		+	-	-
8. 3.'27		+	-	+
20. 4.'27	Non-haemolytic			

The non-haemolytic strain Q11 has retained its non-haemolytic action on blood agar after seven months' cultivation.

During six months the actions on lactose and mannite have remained constant, while on two occasions Q11 has failed and on two occasions has succeeded in fermenting salicin.

N7.

Date	Action on Blood Agar.	Action on Lactose	Mannite	Salicin
9. 8.'26	Non-haemolytic	+	-	-
17. 9.'26		+	-	+*
29. 1.'27		+	-	+
8. 3.'27		+	-	-
20. 4.'27	Non-haemolytic			

*This reaction was found positive after 31 days.

The non-haemolytic strain N7 has retained the non-haemolytic action on blood agar after seven months' cultivation.

During six months the actions on lactose and mannite have remained constant, while the action on Salicin has on two occasions been positive and on two occasions negative.

M6

Date	Action on Blood Agar.	Action on Lactose	Mannite	Salicin
20.10.'26	Non-haemolytic	+	-	+
21. 1.'27		+	-	+

The non-haemolytic strain M6 re-tested as M6 (a) and M6 (b) has remained non-haemolytic in its

action on blood agar during six months.

During five months its action on lactose and mannite sugars has remained constant while its action on salicin has varied.

M6 (a)

Date	Action on Blood Agar.	Action on Lactose	Mannite	Salicin
20. 2.'27	Non-haemolytic	+	-	-
14. 3.'27		+	-	-)
18. 3.'27		+	-	+) *
20. 4.'27	Non-haemolytic			

M6 (b)

Date	Action on Blood Agar.	Action on Lactose	Mannite	Salicin
20. 2.'27	Non-haemolytic	+	-	-
14. 3.'27		+	-	-
18. 3.'27		+	-	+
20. 4.'27	Non-haemolytic			

*I am unable to state whether those reactions were, or were not, obtained on the same batch of medium.

M6 (a) and M6 (b) are strains of M6 isolated on subculture from single colonies.

N2

Date	Action on Blood Agar.	Action on Lactose	Mannite	Salicin
9. 8.'26	Non-haemolytic	+	-	+
28. 8.'26		+	-	+
28. 9.'26		+	-	-*

After eight months the non-haemolytic strain N2 re-tested as N2 (a) and N2 (b) remained non-haemolytic on blood agar.

During seven months of observation the action on lactose and mannite has remained constant while the action on salicin has varied.

N2 (a)

Date	Action on Blood Agar.	Action on Lactose	Mannite	Salicin
2. 2.'27	Non-haemolytic	+	-	+
5. 3.'27		+	-	-*
18. 3.'27		+	-	+
20. 4.'27	Non-haemolytic			

N2 (b)

Date	Action on Blood Agar.	Action on Lactose	Mannite	Salicin
28.10.'27	Non-haemolytic	+	-	-
2. 2.'27		+	-	-
20. 4.'27	Non-haemolytic			

*I have noted here that the growth on salicin is moderately poor.

N2 (a) and N2 (b) are strains of N2 isolated on subculture from single colonies.

N1.

Date	Action on Blood Agar	Action on Lactose	Mannite	Salicin
11.8.'26	Non-haemolytic	+	-	+
21.9.'26		+	-	- *

N1 (a)

Date	Action on Blood Agar	Action on Lactose	Mannite	Salicin
29.1.'27	Non-haemolytic	+	-	+
5.3.'27		+	-	+
18.3.'27		+	-	+
20.4.'27	Non-haemolytic			

N1 (b)

Date	Action on Blood Agar	Action on Lactose	Mannite	Salicin
28.10.'26	Non-haemolytic	+	-	+
29.1. '27		+	-	+

* Growth noted as being moderately poor, as evidenced by fewer organisms per field, and the appearance of an unusual number of diplococci.

The non-haemolytic strain N1 has remained non-haemolytic after eight months' cultivation.

The fermentation reactions on lactose and mannite have remained constant during seven months. Salicin which previously had been fermented, on the second test showed no fermentation. The strain was subcultured on blood agar and strains N1 (a) and N1 (b) isolated in October 1926. These strains in subsequent tests fermented salicin.

DISCUSSION OF RESULTS.

Streptococci and Blood Agar.

The streptococci have been tested on blood agar one for six weeks only and fifteen for periods varying from seven to nine months.

All the strains tested have retained their original action on blood agar.

Streptococci and Carbohydrate Media.

Six Strains or 33% have been variable in their reactions on carbohydrate media and the results of the actions of the streptococci on carbohydrates have been examined from four points of view.

(1) Constancy and the individual sugars (i.e. whether the inconstant strains have shewn their inconstancy towards any one particular sugar, lactose, mannite or salicin.

(2) Constancy of the strains in relation to their sources (i.e. whether inconstant strains are found from all sources skin, throat, blood, faeces.)

(3) Constancy of the reactions in regard to the classified types of streptococcus (i.e. whether any particular type of streptococcus, as classified, shows a tendency towards inconstancy.)

(1) Constancy and the individual Sugars.

All strains have been constant on Lactose media. One strain has shown inconstancy in its reaction to mannite. Strain L3 (from the same original culture as the constant strain L2) developed and maintained the power to ferment mannite - this power being also maintained in a daughter strain L3 (b).

The remaining five strains have all been inconstant on salicin media. One dissimilar negative result of N1 and two of N2 are qualified by a note on comparative feebleness of growth. Strains N7 and Q11 show equal numbers of positive and negative results with all growths satisfactory. This inconstancy has not been that of a power lost or developed by age as in L3 but has been a variability in reaction to salicin. Whereas Broadhurst (39) records salicin as being the sugar on which she found streptococci least variable. Savage (36) and Andrewes and Horder (42) state that the reaction with salicin is one upon which in their opinion too much reliance should not be placed.

(2) The Constancy of the Reactions of the Strains in Relation to their Source.

In examining the sources of these eighteen strains, it is found that eleven strains are from the throat and include five of the six inconstant strains. The high percentage

of throat strains, inconstants on sugars, is in accordance with a statement by Broadhurst (40) that throat strains apparently are more variable in their reactions to sugars than strains from any other source.

(3) The Constancy of the Reactions in regard to the Classified Types of the Streptococcus.

On examining the results of the actions on carbohydrates in relation to the type of streptococcus five of the six inconstant strains are noted to be streptococcus mitis. (Holman (33) does not record a constancy test for streptococcus mitis).

(4) The Constancy of the Reactions on Carbohydrate Media in regard to whether the Strains are Haemolytic or non-haemolytic.

The outstanding feature of the constant strains is that they include all the haemolytic strains examined. In no case among the haemolytic strains has a variation from the original finding been observed. A second noteworthy feature of these strains is that excepting N3 the actions of one strain on carbohydrates can be compared with the actions of another strain derived from the same original culture. Thus I_3 and I_4 are from the same spleen post mortem, K2 and K3 are from the same skin lesion, Q6, Q20 and Q60 are from the same infected tonsils. The strains have not only maintained their original fermentative

abilities, but other strains from the same source and isolated at the same time have shewn and maintained similar fermentative powers. It is of interest in this connection and in connection with the constancy of throat strains, that of the six constant throat strains, two were examined only over short periods, and the remaining four were haemolytic.

Among the ten non-haemolytic streptococci only four strains M4, N5, N3 (b) and L2 have shewn no variation in reaction - and the former two were observed only over five and six weeks respectively.

The non-haemolytic streptococci have thus not shewn the constancy displayed by the haemolytic series. Such a finding is published by Clawson (37) and by Hopkins and Lang (38).

CONCLUSIONS.

(1) The Action of streptococci on blood agar is not influenced by routine laboratory subculturing.

(2) Routine laboratory subculturing does not influence the action on carbohydrates of haemolytic streptococci.

(3) The non-haemolytic strains of streptococci are not so constant in their fermentative reactions as the haemolytic.

(4) Routine laboratory subculturing does not influence the fermentation reactions of lactose and mannite.

(5) The action of non-haemolytic throat strains of streptococci on salicin tends to be unreliable.

-----oOo-----

BIBLIOGRAPHY.

-oOo-

1. Libman - Journ. Med. Research 1901, VI p.84.
2. Gordon - Lancet 1905, II p. 1400.
3. Crowe - Journ. Path. & Bact. 1921 vol. 24, p. 362.
4, Brown & Smith - Journ. Med. Research, 1915, vol.31, p.467.
5. J.H. Dible - Journ. Path & Bact. 1921, vol. 24, p. 32.
6. Andrewes & Horder - Lancet 1906, II pp. 708,775, 852.
7. Winslow - Journ.Infec. Dis. 1912, vol. X, p. 293.
8. Broadhurst - Journ.Infect.Dis. 1913, vol. XIII, pp. 404-407.
9. Avery & Cullen - Journ.Exper.Med. 1919, XXIX, p. 233.
11. J. Howard Brown - Journ. Exper. Med. 1920, XXXI p. 46.
12. Andrewes & Horder - Lancet 1906, II p. 710.
13. Floyd & Wolbach - Journ.Med. Research 1914, XXIX, p. 513.
14. Lyall - Journ. Med. Research 1914, XXX, p. 511.
15. Holman - Journ. Med. Research 1916, XXXIV, p. 440.
16. Blake - Journ. Med. Research 1917, XXXVI, p. 99.
17. Ritchie - Lancet 1908, II p. 376.
18. D.H. Bergey - Journ.Med. Research 1912-13, XXVII, p. 70.
19. David John Davis - Journ. Infect. Dis. 1913, XII p. 389.
20. Kliger - Journ. Infect.Dis. 1915, XVI, p. 331.
21. Beattie & Yates - Journ. Path. & Bact. 1911-12, XVI p.255.
22. E.W.A. Walker - Journ. Path. & Bact. 1911, XV, p.124.
23. H. Warren Crowe - Journ.Path & Bact. 1924, XXVII, p. 450.

24. E.C. Rosenow - Journ. Infect. Dis. 1914, XIV, p.V.

25. Floyd & Wolbach - Journ. Med. Research 1914, XXIX, p. 510.

26. McLeod - B.M.J. 1921, vol. II p. 791.

27. Beattie & Yates - Journ. Path & Bact. 1911-12,XVI, p.253.

28. Lyall - Journ. Med. Research 1914, XXX p. 487.

29. Andrewes & Horder - Lancet 1906, II p. 710.

30.Gordon - Lancet 1905, II p. 1403.

31. Andrewes & Horder - Lancet 1906, II p. 709.

32. Holman - Journ. Infect. Dis. 1914, XV, pp. 210-11.

33. Holman - Journ. Med. Research 1916, XXXIV, pp. 419-421.

34. Holman - Journ. Infect. Dis. 1914, XV, pp. 297-8.

35. Holman - Journ. Infect. Dis. 1914, XV, pp. 227-228.

36. Savage - Supplement Local Government Board Reports, 1906, 1907, p. 251.

37. B.J. Clawson - Journ. Infect. Dis. 1920, 26, p. 115.

38. Hopkins & Lang - Journ. Infect. Dis. 1914, XV, p. 74.

39. Broadhurst - Journ. Infect. Dis. 1915 XVII, p. 285.

40. Broadhurst - Journ. Infect.Dis. 1915, XVII, p. 286.

41. Broadhurst - Journ. Infect. Dis. 1915, XVII, p. 284.

42. Andrewes & Horder - Lancet 1906, II, p. 710.

--------oOo--------