INFLUENCE OF VARIATIONS OF MEDIA ON ACID PRODUCTION BY STREPTOCOCCI.

By F. S. JONES, V.M.D.

(From the Department of Animal Pathology of The Rockefeller Institute for Medical Research, Princeton, N. J.)

(Received for publication, May 5, 1920.)

In an attempt to differentiate species of organisms closely resembling each other, the fermentation of carbohydrates and other substances plays an important part. When the fermentation characters of streptococci were first studied indicators such as litmus were employed which revealed only qualitative changes. Later quantitative determinations of acid by titration against solutions of alkali came into use.

More recently Clark and Lubs[1] have employed the hydrogen ion concentration method as an aid in the differentiation of closely allied species of organisms. Several have applied it for determination of acid production by streptococci. The advocates of the newer method point out that it is more accurate than titration since it indicates only free acids. The term "limiting or final hydrogen ion concentration" has come into use, since it indicates the maximum acidity or alkalinity produced by a given organism. Thus Avery and Cullen[2] have shown that human streptococci grown in veal infusion bouillon containing 1 per cent dextrose attained a limiting hydrogen ion concentration of 5.0 to 5.2. The bovine group produced more acid, 4.3 to 4.5. Ayers, Johnson, and Davis[3] some time previously had grown human hemolytic streptococci in a desiccated yeast-peptone-dextrose medium, one part of each per 100 parts of water, and found that the bulk of their strains reached a final hydrogen ion concentration of 5.4 to 6.0; 5.6 seems to have been the average. Smillie,[4] who used 1 per cent dextrose in fermented veal infusion bouillon, records the figures pH 5.1 to 5.4 for a few human strains. Brown[5] observed a limiting acidity of pH 5.1 to 5.4 for human streptococci grown in plain bouillon containing 1 per cent dextrose.

---

[1] Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 1.

[2] Avery, O. T., and Cullen, G. E., *J. Exp. Med.*, 1919, xxix, 215.

[3] Ayers, S. H., Johnson, W. T., and Davis, B. J., *J. Infect. Dis.*, 1918, xxiii, 290.

[4] Smillie, W. G., *J. Infect. Dis.*, 1917, xx, 45.

[5] Brown, J. H., *J. Exp. Med.*, 1920, xxxi, 35.

Fennel and Fisher[6] record the acid limit of *Streptococcus hæmolyticus* as pH 4.5. Whether these figures include both the human and bovine varieties is not stated.

Since considerable variation in acid production had been observed by various workers it seemed possible that the differences might be the result of variations in the media. Broadhurst[7] brought out the difference in the amount of titratable acid produced by streptococci grown in broth prepared from meat and meat extract. Streptococci grown in the former media produced two or three times as much acid as those grown in meat extract media. Many have observed the marked increase in the amount of titratable acid when more than 1 per cent of peptone was used in sugar broths. The writer has frequently noted that streptococci grown in fermented bouillon containing 1 per cent dextrose to which sterile serum had been added invariably produced more titratable acid than cultures in the same media which did not contain serum. A small number of tests also showed that the hydrogen ion concentration was often greater in the dextrose serum broth. The results, then, were not readily explicable on the assumption that the buffer activity of the serum was responsible for the increase in the acidity. It seemed desirable to note the effect of variations of the medium upon acid production.

Veal infusion was prepared from the flesh of a calf 1 day old. It is customary in this laboratory to add two parts of water to one part of the ground flesh. The infusion was divided. To one portion 1 per cent of peptone (Fairchild's) and 0.5 per cent of sodium chloride were added and the reaction was adjusted to + 0.8 (pH 7.6). The remainder of the infusion was strained and inoculated with a young culture of *Bacillus coli* and permitted to ferment at 38° C. for 18 hours. After straining through cotton and gauze, the usual quantity of peptone and salt was added to the larger portion of the filtrate. The reaction was adjusted to + 0.8 (pH 7.6). To a smaller portion of the fermented infusion 2 per cent of peptone and the usual amount of sodium chloride were added. This then provided three types of bouillon prepared from the same materials, unfermented broth and fermented bouillon containing 1 and 2 per cent of peptone respec-

[6] Fennel, E. A., and Fisher, M. B., *J. Infect. Dis.*, 1919, xxv, 444.
[7] Broadhurst, J., *J. Infect. Dis.*, 1913, xiii, 404.

tively. The broths were tubed in amounts of 13 cc. After sterilization under pressure, 1 cc. of a sterile 13 per cent solution of dextrose was added to each. The columns of liquid in the tubes ranged from 6 to 6.5 cm. in height. To half of the tubes of fermented and half of the tubes of unfermented bouillon, 0.5 cc. of sterile horse serum per tube was added. The 2 per cent peptone-dextrose fermented broth was used without other additions.

All cultures employed in the experiment produced hemolysis (beta) in agar plates containing 8 per cent of defibrinated horse blood. All the human streptococci[8] had been grown on artificial media for 2 or more years. All had been isolated from diseased conditions. The bovine strains were isolated by the writer. Five were from cases of mastitis. Two were found in market milk, but were identical in all respects with mastitis streptococci. The equine streptococci, with one exception, were isolated from the nasal mucosa and pharynx of horses suffering from influenza. Equine Streptococcus H.A. 2 was cultivated from an abscess. The low acid-producing streptococci were isolated from market milk. Recent investigations[9] have shown that these may be carried in small numbers in apparently normal udders. Strains M.J. 1 and M. 53 were isolated in 1917. The others were first cultivated in the summer of 1918.

Each tube was inoculated with 0.1 cc. of an 18 hour broth culture. All were incubated at 38°C. for 10 days, when hydrogen ion determinations[10] and titrations against 0.05 N sodium hydroxide were made. Maximum growth was always obtained in the media containing serum. The plain and fermented bouillon cultures were about as vigorous as those observed in the 2 per cent peptone medium. The low acid-producing streptococci from milk grew poorly in media without serum. Serum frequently changes the character of the growth. Strains which grow only at the bottom of tubes in the plain broth frequently produce a marked turbidity throughout the serum medium.

[8] The writer is indebted to Dr. J. Howard Brown, of this Department, for Cultures 32, S.H., S. 8, and 40. Dr. O. T. Avery, of the Hospital of The Rockefeller Institute, supplied Cultures 1, 20, and 24.

[9] Jones, F. S., *J. Exp. Med.*, 1920, xxxi, 347.

[10] Dr. P. E. Howe, of this Department, prepared the standards and indicators for this experiment.

In Tables I to IV the acid production by the different groups of streptococci is given. The figures under the titration columns represent the actual acidity reached in the tubes.

TABLE I.

*Acid Production by Human Streptococci in Plain and Fermented Broths.**

Strain No.	Fermented bouillon.		Fermented bouillon and serum.		Fermented bouillon containing 2 per cent peptone.		Plain bouillon.		Plain bouillon and serum.	
	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.
		per cent		*per cent*		*per cent*		*per cent*		*per cent*
32	5.5	3.6	5.2	4.2	5.7	4.5	5.1	4.2	5.0	4.8
S.H.	5.5	4.2	5.2	4.5	5.5	5.0	5.2	4.0	4.9	4.6
S. 8	5.8	3.9	5.2	4.1	6.0	3.9	5.6	3.2	4.9	4.2
40	5.2	4.2	5.0	4.6	5.4	4.9	5.1	4.1	5.0	4.7
1	5.8	3.5	5.2	4.2	5.8	3.9	5.5	3.3	5.0	4.4
20	5.7	3.1	5.2	4.0	5.7	3.9	5.2	3.2	5.1	4.4
24	5.7	3.1	5.1	4.2	5.7	4.3	5.1	3.7	4.9	4.5

* All the media contained 1 per cent dextrose.

TABLE II.

Acid Production by Bovine Streptococci in Plain and Fermented Broths.

Strain No.	Fermented bouillon.		Fermented bouillon and serum.		Fermented bouillon containing 2 per cent peptone.		Plain bouillon.		Plain bouillon and serum.	
	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.
		per cent		*per cent*		*per cent*		*per cent*		*per cent*
B.M. 1	4.6	6.1	4.6	6.2	4.6	7.0	4.6	5.7	4.6	5.9
C. 59	4.8	5.7	4.8	6.2	5.0	7.0	4.6	6.2	4.6	6.1
B.M. 24	4.9	5.4	4.8	6.1	4.9	6.7	4.6	5.8	4.5	5.7
M. 26	4.8	5.5	4.7	6.3	4.8	7.1	4.6	5.8	4.5	5.7
" 43	4.7	6.0	4.6	6.5	4.7	7.2	4.6	5.9	4.6	5.9
C. 53	4.8	5.3	4.7	6.0	4.8	6.8	4.6	5.5	4.5	5.9
" 67C	4.8	6.2	4.8	6.5	4.8	7.4	4.6	6.0	4.6	5.9

Curves (Text-figs. 1 and 2) constructed from averages of Tables I to IV reveal considerable differences in acid formation in the various media. This is particularly true in the instance of the human and low acid-producing streptococci from milk. It will be observed that

these organisms grown in media low in nutritive material (fermented and plain broth), even though the media contain sufficient carbohydrate, fail to produce as much acid as when a richer medium is employed. The addition of serum, then, not only increases the titratable acid but the ionized acid as well. Thus in the richest medium

TABLE III.

Acid Production by Equine Streptococci in Plain and Fermented Broths.

Strain No.	Fermented bouillon.		Fermented bouillon and serum.		Fermented bouillon containing 2 per cent peptone.		Plain bouillon.		Plain bouillon and serum.	
	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.
		per cent		*per cent*		*per cent*		*per cent*		*per cent*
In. 22	5.1	4.4	5.5	4.4	5.0	5.5	4.9	4.4	5.1	4.6
H.A. 2	5.1	4.3	5.5	3.2	5.1	5.3	4.7	4.4	5.0	4.6
In. 49	5.1	4.5	5.3	5.0	5.1	5.7	4.9	4.4	5.1	4.8
" 14	5.1	3.9	5.4	4.4	5.0	4.8	4.9	3.9	5.1	4.6
" 2	5.1	4.7	5.4	4.2	5.1	5.6	4.9	4.6	5.1	4.5

TABLE IV.

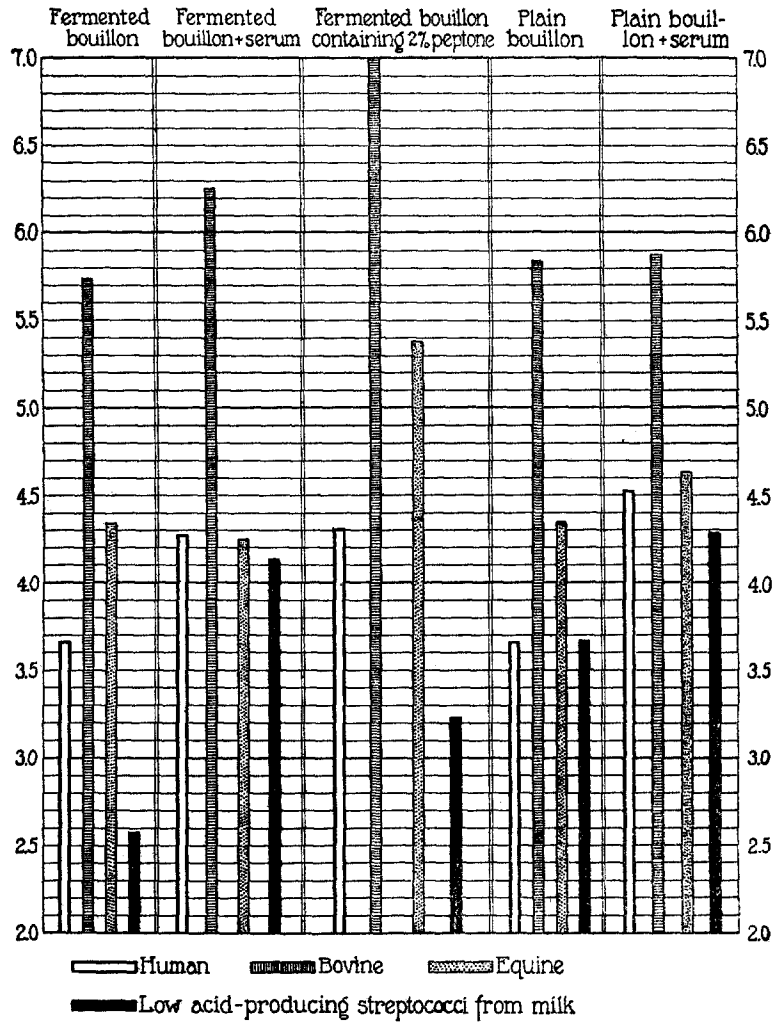
Low Acid-Producing Streptococci from Milk.

Strain No.	Fermented bouillon.		Fermented bouillon and serum.		Fermented bouillon containing 2 per cent peptone.		Plain bouillon.		Plain bouillon and serum.	
	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.	pH	Titration.
		per cent		*per cent*		*per cent*		*per cent*		*per cent*
M.J. 1	6.3	2.6	5.4	4.1	6.5	3.1	5.2	4.0	5.2	4.0
M. 53	6.3	2.7	5.8	3.5	6.3	2.4	5.8	3.0	5.2	4.1
B.M. 30	6.6	2.0	5.2	4.1	5.9	3.7	5.1	4.0	5.1	4.3
" 22	5.9	3.1	5.1	4.6	6.3	3.7	5.5	3.4	5.1	4.6
" 60	6.3	2.4	5.1	4.4	6.3	3.2	5.5	3.9	5.1	4.4

(dextrose bouillon and serum) the maximum acid production is reached. The sharpest differences are brought out by the low acid-producing milk streptococci; here the greatest increases in acid formation are seen in the serum media. The fermented broth containing 2 per cent peptone apparently offers for the human and low acid-producing streptococci no more favorable media for acid production than that

containing 1 per cent peptone. One would expect the titratable acid to increase on account of the great buffer activity of peptone.

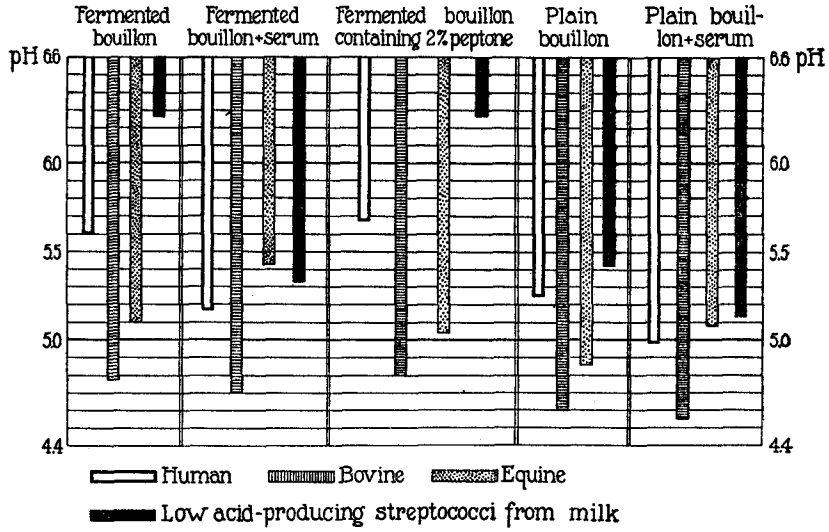
The bovine streptococci follow a somewhat similar curve. Apparently organisms of this type are able to utilize carbohydrate to



TEXT-FIG. 1. Titratable acidity produced by human, bovine, and equine streptococci and low acid-producing streptococci from milk when grown in various media.

a greater degree in media of lower nutritive value. The titratable acidity varies over a considerable latitude; the buffer effect of the 2 per cent peptone is marked. On the whole, there is more acid produced in the serum media.

The reverse is true of the equine streptococci. The organisms apparently produce more acid in media which do not contain serum. Broth containing 2 per cent peptone affords a medium equally as good for acid production as either of the other bouillons to which serum was not added. Two possible explanations for the failure of the



TEXT-FIG. 2. Limiting or final hydrogen ion concentration of human, bovine, and equine streptococci and low acid-producing streptococci from milk when grown in various media.

serum to increase the production of acid suggest themselves. The equine streptococci may not require serum; peptone and meat extracts may be sufficient for all purposes. On the other hand, normal horse serum may contain some substance antagonistic to growth. It is admitted that all strains grew well in the serum media.

The experiment was repeated with the same cultures, but with broths made from the flesh of different calves. When bouillon prepared from the flesh of a calf 6 weeks old was employed much the same results were obtained.

DISCUSSION.

The results obtained readily show that the limiting hydrogen ion concentration may be influenced by differences in the media. The question naturally arises as to the final or limiting hydrogen ion concentration reached by an organism. From present knowledge it may be defined as the acid production in a given medium which finally limits the growth of the organism. That such figures vary over a considerable latitude must be admitted, since human streptococci in one medium may produce acid to the value of pH 5.6 and in another to pH 4.9. Avery and Cullen's figures represent the maximum acid production for human (pH 5.0 to 5.2) and bovine (pH 4.3 to 4.5) streptococci in a medium most favorable for growth. Ayers, Johnson, and Davis' figures (5.5 to 6.0), on the other hand, are given as the final hydrogen ion concentration in a medium of low nutritive value for pathogenic streptococci. While these data were being gathered together the paper of H. Jones[11] appeared. He obtained even greater variations than those recorded in the preceding protocols. He showed that a culture of *Streptococcus hæmolyticus* when grown in glucose broth produced acid to the value of pH 5.11, but when ascitic fluid was added to the bouillon an acidity of pH 4.63 was reached. The increase in acidity failed to vary with an increase in the amount of enriching material. The same limiting hydrogen ion concentration was reached in media to which varying amounts (3 drops to 3 cc.) of ascitic fluid had been added. H. Jones also observed that the initial reaction of the media may influence the limiting hydrogen ion concentration. Thus in concluding it is pointed out that the limiting hydrogen ion concentration of an organism should be defined in terms of media composition, the initial reaction, and other conditions which may favor or hinder abundant growth.

Clark and Lubs[12] noted that methyl red may be destroyed in a short time by active cultures of *Bacillus coli*. Others had observed the same for nitrifying bacteria. Certain streptococci, especially those from sour milk, exhibit this activity to a marked degree while in the active growth phase. The indicator begins to fade within 5 or 10 minutes and at the end of an hour is completely decolorized.

[11] Jones, Horry, *J. Infect. Dis.*, 1920, xxvi, 160.

[12] Clark, W. M., and Lubs, H. A., *J. Bacteriol.*, 1917, ii, 191.

The advocates of the exclusive application of the hydrogen ion concentration method to bacteriological study have frequently pointed out the unreliability of titration. In studying the tables and charts it will be observed that the differences in titratable acidity are clear-cut. Even the addition of 2 per cent peptone with its buffer effect still reveals great differences in the amount of acid formed by the different groups of organisms. From these experiments one is inclined to believe that titration is equally as satisfactory as the newer method for the study of the fermentative activity of streptococci.

The greatest differences in acid production are brought out in a relatively unfavorable medium, such as dextrose fermented broth containing 1 per cent peptone.

SUMMARY.

The results of variations in acid production in 1 per cent dextrose fermented and unfermented veal broth modified by the addition of 4 per cent of horse serum or 2 per cent of peptone have been recorded. Human streptococci and a group of low acid-producing streptococci from milk produce less acid in the simpler broths (fermented and unfermented). 2 per cent peptone fails to increase the amount of acid produced by these two groups.

The bovine streptococci act much the same as those of human origin. The equine streptococci apparently do not require serum in addition to carbohydrate since they tend to produce less acid in serum media.

The following figures indicate the average minimum and maximum acid production of the various streptococci under the conditions set forth in the experiment.

Human: pH 4.97 to 5.66; titratable acidity 4.51 to 3.66.

Bovine: pH 4.56 to 4.77; titratable acidity 7.0 to 5.74.

Equine: pH 4.86 to 5.42; titratable acidity 5.38 to 4.24.

Low acid-producing streptococci from milk: pH 6.28 to 5.14; titratable acidity 2.56 to 4.28.