HYDROGEN ION CONCENTRATION OF CULTURES OF PNEUMOCOCCI OF THE DIFFERENT TYPES IN CARBOHYDRATE MEDIA.

By O. T. AVERY, M.D., and GLENN E. CULLEN, Ph.D.

(*From the Hospital of The Rockefeller Institute for Medical Research.*)

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Knowledge of the physiological activities and immunological characters of bacteria serves not merely the purposes of systematic classification, but contributes to a fuller understanding of the problems of infectious disease. The correlation of these apparently independent characters with pathogenicity and with the occurrence and distribution of recognizable types under a wide variety of environmental conditions is essential to the proper interpretation of the phenomena of infection.

Of the physiological characters of bacteria the fermentation of certain carbohydrates by some, and the inability of other closely related organisms to utilize the same substances, are relatively fixed characteristics of sufficient constancy to warrant their use as a basis for differentiation. In fact, within a given species these biochemical reactions are of considerable value in distinguishing type relationships. The application of this principle in the classification of the typhoid-dysentery group affords an illustration of the value of the biochemical method in determining the existence of certain varieties of these organisms and their relation to enteric infections. On the other hand, it has been possible in the case of pneumococcus to acquire knowledge of the occurrence of specific types and their relation to disease, chiefly by a study of the immunological characters of these organisms, since their biochemical activities are apparently possessed in common by all.

The measure of acid production by bacteria has been the determination of the amount of titrable acidity produced in a medium containing a known concentration of carbohydrate. This method is now recognized as inadequate. Recently the colorimetric determination

of the hydrogen ion concentration has been adapted to bacteriological requirements and affords an additional method for measuring this phase of bacterial metabolism. Moreover, in addition to the selective fermentation of certain substances by bacteria, the maximum acidity that can be tolerated by a given organism is apparently a biologic constant. Accordingly, the determination of the hydrogen ion concentration at which bacterial growth ceases has been utilized as a differential method. In the case of a culture growing in a medium containing an excess of fermentable substance this limiting reaction is spoken of as the final hydrogen ion concentration. This phenomenon has been utilized by Clark (1) and Clark and Lubs (2) in the differentiation of the colon-*aerogenes* group, and by Ayers (3), Ayers, Johnson, and Davis (4), and Avery and Cullen (5) in the recognition of differences between hemolytic streptococci of human and bovine origin.

The present paper presents the results, obtained under the experimental conditions defined, of a study of the influence of the concentration of dextrose on the final hydrogen ion concentration of broth cultures of pneumococcus, the rate of acid production, the optimum and limiting hydrogen ion concentration for initial growth of pneumococcus, the final hydrogen ion concentration of cultures of pneumococcus in carbohydrate media, and a comparison of these physiological functions with the specificity of the immunological type characters of these organisms. In addition, some information has been acquired concerning the factors governing growth of pneumococcus when reinoculated into filtrates of cultures of the same and different types.

EXPERIMENTAL.

Source of Material.—Thirty-nine strains of pneumococcus comprising representatives of the various immunological types were studied. In the majority of instances these cultures were isolated directly from the blood or sputum of patients suffering from lobar pneumonia. Some of the strains were tested immediately upon isolation, others after years of cultivation on artificial media. Most of the strains were pathogenic for white mice and had been passed through these animals to enhance virulence. Cultures used for inoculation of the test medium were grown for 18 hours at 37°C. in plain meat infusion broth of pH

7.8. The serological methods used for the determination of types of pneumococcus were the same as those already described (6).

Determination of Hydrogen Ion Concentration.—The colorimetric method of determining hydrogen ion concentration was chosen because of its convenience and simplicity. The principle and details of this method have been so thoroughly reviewed that it is unnecessary to repeat them here (7). In the present work the two principal sources of error, color of the medium and turbidity of the culture, have been avoided by first diluting the medium and then compensating for the color by Walpole's comparator method of superimposing the color of the medium upon that of the indicator.

The hydrogen ion concentrations are expressed in the customary manner as pH values; that is, the negative exponent to the base 10 of the normality.

Duplicate tubes containing 5 cc. of the medium or culture were diluted to 15 cc. with redistilled water, four drops of indicator solution were added to one tube, and the tube was compared in a comparator block with a standard solution containing exactly the same amount of indicator. The standard solutions of known hydrogen ion concentration were prepared for the range pH 8.6 to 5 from standard phosphates by Sörensen's technique, and for the range pH 5.8 to 4.6 from acetic acid-sodium acetate mixtures by Walpole's directions. The accuracy of these standard solutions was verified by the hydrogen electrode.

The indicators used were:

	per cent
pH 8.6–8.0, *o*-cresol sulfonephthalein	0.04
pH 8.0–6.8, phenolsulfonephthalein (phenol red)	0.04
pH 6.8–5.8, bromocresolsulfonephthalein, saturated water solution.	
pH 5.8–4.6, methyl red	0.04

Sterilization of the Medium Containing the Test Substance.—In studying fermentative activity of bacteria the manner in which the medium containing the test substance is sterilized is of first importance. If a sugar, such as sucrose, is added to the slightly alkaline broth and sterilized in an Arnold sterilizer or boiled for a long time, the sugar will be hydrolyzed. It seemed best, therefore, to sterilize by boiling a concentrated solution of the substance in water. Enough of this con-

centrated solution is then added to sterile broth to the desired dilution. 10 minutes in boiling water has sufficed in most cases to effect sterilization. When the medium has been handled many times, in adjusting to different reactions, further sterilization has been considered necessary. Additional sterilization is indicated in the protocol.

Influence of Sugar Concentration upon the Final Hydrogen Ion Concentration.—In studying the sugar-fermenting property of any organism it is, of course, essential to determine the influence of varying concentrations of sugar upon the final reaction. In order to do this for pneumococcus the following experiment was carried out.

To sugar-free broth of known hydrogen ion concentration sterile 20 per cent dextrose solutions (boiled 10 minutes) were added in sufficient amounts to make the desired concentration. The medium was incubated over night to test sterility, and 50 cc. portions were inoculated with 0.1 cc. of an 18 hour plain broth culture of pneumococcus. The hydrogen ion concentration of the medium was determined before the inoculation and after 24 and 48 hours incubation (Table I).

TABLE I.

Influence of the Concentration of Dextrose on the Final Hydrogen Ion Concentration of Pneumococcus Cultures.

50 cc. of sugar-free* broth, pH 7.5, inoculated with 0.1 cc. of 18 hour plain broth culture of Pneumococcus Type II (Strain F 149).

Dextrose.	Hydrogen ion concentration.	
	24 hrs.	48 hrs.
per cent	*pH*	*pH*
Broth control (uninoculated).	7.5	7.5
0	7.3	7.2
0.1	6.5	6.5
0.2	6.0	6.0
0.4	5.1	5.0
1.0	5.0	5.0
2.0	5.0	5.0
4.0	5.0	4.9

* In the preparation of sugar-free broth the meat infusion, before the addition of peptone, is fermented by *B. coli* for 18 to 24 hours.

It is evident that 0.4 per cent dextrose furnished sufficient acid to bring the medium to the limiting hydrogen ion concentration, but that excess of sugar up to at least 4 per cent has no influence upon the final reaction. 1 per cent, therefore, was chosen as sufficient for all routine fermentations. This agrees closely with a similar experiment with *Streptococcus hæmolyticus* (5).

Rate of Acid Production of Pneumococcus in 1 Per Cent Dextrose Broth.—It was to be expected that the rate of acid production would be dependent upon the size of the inoculum, but it was desirable to compare the rate curve in dextrose broth with that in plain broth. Moreover, it was necessary to determine the time required for the attainment of the final hydrogen ion concentration. In determining the rate a massive inoculum was used to bring the experiment within 1 day (Table II).

TABLE II.

Rate of Acid Production by Pneumococcus in Dextrose Broth.

100 cc. of sugar-free broth plus dextrose to 1 per cent, inoculated with 0.5 cc. of an 18 hour plain broth culture of pneumococcus, and incubated at 37°C.

Pneumococcus.	1 per cent dextrose broth. Initial hydrogen ion concentration.	Hydrogen ion concentration.					
		1½ hrs.	4 hrs.	6 hrs.	8 hrs.	9 hrs.	27 hrs.
	pH	*pH*	*pH*	*pH*	*pH*	*pH*	*pH*
Type I	7.75	7.7	7.3	6.5	5.6	5.3	5.0
" II	7.9	7.9	7.5	6.8	5.7	5.3	4.8

No change in reaction of the dextrose broth occurred during the period of initial lag, then acid was produced at a rapid and constant rate until the final reaction was reached—in this case a pH 4.8 to 5.

With the usual inoculum employed in these experiments, 0.1 cc. of an 18 hour culture to 50 cc. of broth, the final hydrogen ion concentration is attained within 24 hours. An occasional culture will show a further increase in acidity of not more than 0.1 pH after 2 to 7 days incubation, but such changes are within the limits of experimental error. Table III furnishes an illustration of this fact. In a few instances in this experiment 18 hours was not sufficient time for the

attainment of the final reaction, but no apparent changes took place after 30 hours. As a routine, therefore, the cultures were read after 24 or 48 hours incubation at 37°C.

TABLE III.

Relation of the Type of Pneumococcus to the Final Hydrogen Ion Concentration in Sugar Broth.

75 cc. portions of 1 per cent sugar broth having an initial reaction of pH 7.8 were inoculated with 0.1 cc. each of an 18 hour plain broth culture of pneumococcus of different types.

Pneumococcus.		Hydrogen ion concentration.					
		Dextrose.			Lactose.		
Type.	Strain.	18 hrs.	30 hrs.	5 days.	18 hrs.	30 hrs.	5 days.
		pH	*pH*	*pH*	*pH*	*pH*	*pH*
I	F 55	5.1	5.1	5.1	5.1	5.1	5.1
I	146	5.0	5.1	5.0	5.0	5.0	5.0
II	46	4.9	5.0	5.0	5.0	5.0	5.0
II	F 149	5.2		5.1	5.1	5.1	5.1
IIa	J	5.8	5.0	5.0	5.3	4.9+	5.0
IIb	W	5.1	5.2	5.2	5.3	5.1	5.1
III	A 66	5.1	5.05	5.05	5.1	5.0	5.1
III	F 104	5.0	5.0	5.0	5.1	5.0	5.1
IV	L F	5.0	5.0	4.9	5.1	5.2	5.1
IV	L A	5.0	5.2	5.2	5.1	5.2	5.2

Relation of the Type of Pneumococcus to the Final Hydrogen Ion Concentration of Cultures in Sugar Broth.—In order to determine whether the differences in antigenic properties of the specific types of pneumococcus had any influence on the final reaction in sugar medium, two strains of each of the four types, as well as strains representing subgroups of Type II were inoculated into broth containing 1 per cent dextrose or lactose. It is evident from Table III that the final pH of all strains of pneumococcus was between pH 5.2 and 4.9, with the majority between pH 5.0 and 5.2. The differential characters as determined by immunological reactions were not evident from the final hydrogen ion concentrations. It is of significance that the final hydrogen ion concentration of cultures of pneumococcus is exactly the same as the final reaction of pathogenic hemolytic streptococci of

human type. This is also brought out in Table IV where the results of all the strains used in these and succeeding experiments are summarized by types. This table represents 54 determinations on 39 strains. Duplicate determinations in individual experiments are not included in this table.

TABLE IV.

Final Hydrogen Ion Concentration of Pneumococcus in Dextrose Broth. Summary of 54 Determinations on 39 Strains.*

Type I.		Type II.		Type III.		Type IV.	
Strain.	Hydrogen ion concentration.	Strain.	Hydrogen ion concentration.	Strain.	Hydrogen ion concentration.	Strain.	Hydrogen ion concentration.
	pH		*pH*		*pH*		*pH*
I	5.2	II	5.0	A 66	5.0	L F	5.0
I	5.0	II	4.9	A 66	5.1	L A	5.2
I	5.1	F 149	5.1	F 104	5.0	F 194	5.1
F 55	5.1	F 149	5.1	F 104	4.8	X 47	5.2
E 22	5.1	F 149	5.1	D 40	5.0	E 190	5.0
F 152	5.0	F 149	5.1	D EH	4.9	E 190	4.8
D 6	5.0	F 149	5.0	U	5.1	E 117	5.0
D 46	5.0	F 149	5.0	E 84	5.0	E 157	5.0
D 46	4.8	F 149	5.1	C 28	5.0	E 121	5.2
D 46	5.0	F 149	5.3	E 111	4.9	D 107	5.0
16,887	5.0	D 39	5.0	G	5.0	Sch.	4.9
16,867	5.0	IIa J	5.0	E 127	5.0		
F 169	5.0	IIb W	5.0	A	4.9		
		IIc A	5.0				
		IIc 50	5.0				
		II atypical, F 150	5.0				
		II " F 154	5.1				
Total 13		17		13		11	
Average.....	5.0		5.0		4.9		5.0

* Repetition of strains in the above table represents observations made in different experiments.

It is evident from the preceding experiment that with dextrose and lactose there is no type difference in acid production. A number of other carbohydrates fermentable by pneumococcus have been tested.[1]

[1] The substances used were either Kahlbaum's reagents or were those prepared by Mr. E. P. Clark of The Rockefeller Institute for Medical Research.

The media were prepared by placing solutions or suspensions of the substance in boiling water for 10 minutes, and adding the required amount to 75 cc. of media. The media were then heated in the Arnold sterilizer once for 20 minutes, and incubated to test sterility. They were inoculated with 0.1 cc. each of 18 hour plain broth cultures of pneumococcus representing the four types. Samples were removed for pH determinations after 48 hours and 7 days. Since 7 day readings are practically identical with 48 hour readings, only the latter are given (Table V).

TABLE V.

Final Hydrogen Ion Concentration of Pneumococcus in Various Carbohydrate Media.

75 cc. of sugar-free broth containing 1 per cent carbohydrate, initial pH 7.5, were inoculated with 0.1 cc. of an 18 hour plain broth culture and incubated 48 hours at 37°C.

Test substance.	Uninoculated control.	Pneumococcus type.			
		I (Strain F 152).	II (Strain F 149).	III (Strain F 104).	IV (Strain E 190).
	pH	*pH*	*pH*	*pH*	*pH*
Maltose	7.5	5.0	5.0	4.9	5.0
Saccharose	7.5	5.0	5.1	5.0	5.0
Lactose	7.5	5.0	5.0	5.0	4.9
Galactose	7.3	5.1	5.1	5.1	4.8
Raffinose	7.5	5.0	5.1	5.1	5.1
Dextrose	7.5	5.1	5.1	4.8	4.8
Inulin	7.5	5.1		5.1	5.1

From the results presented in Table V it is evident that in media containing sufficient fermentable carbohydrate, growth of pneumococcus continues until a final hydrogen ion concentration of about pH 5 is reached. Apparently this acidity is sufficient in itself to stop growth and the organisms die in the products of their own metabolism. Cultures of pneumococci with all the carbohydrates which were fermentable under the conditions used, namely maltose, saccharose, lactose, galactose, raffinose, dextrose, and inulin, gave identical results in the rate of reaction change and final hydrogen ion concentration (pH 5) attained. Further, the different immunological types of pneumococcus, in a limited number of strains studied, behave alike in fermenting these carbohydrates.

Optimum Hydrogen Ion Concentration for Growth of Pneumococcus.—Work previously reported from this laboratory (8) showed that the optimum hydrogen ion concentration for the growth of pneumococcus was about pH 7.8, and that the hydrogen ion concentrations within which growth could be initiated were between pH 7.0 and 8.3. It was desirable to determine whether the optimum pH in sugar broth was the same as that in plain broth and also to determine for dextrose broth the upper and lower hydrogen ion concentrations beyond which initial growth does not occur.

50 cc. portions of plain broth containing 0.2 per cent phosphate and the same broth containing 1 per cent dextrose were adjusted to the desired reactions by addition of either sodium hydroxide or hydrochloric acid. The media were placed in boiling water for 10 minutes, and incubated for 48 hours to test sterility. They were then inoculated with 0.25 cc. of an 18 hour culture of Pneumococcus Type I (Strain F 169). After 6 hours, estimation of growth and pH determinations were made, and a portion was autoclaved for determination of turbidity by the nephelometer method (Kober instrument). Essentially the same procedure was used as that described by Dernby and Avery (8), except that it seemed unnecessary to estimate the number of organisms per cubic centimeter, since it is in reality a function of the size of the inoculum. The readings on the nephelometer were expressed, therefore, as percentages of the heaviest growth (Table VI).

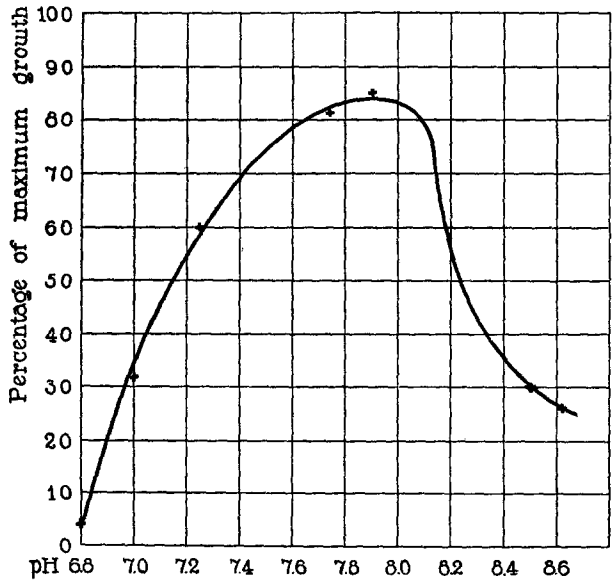
This method is entirely satisfactory with plain broth, and the results are in agreement with those of Dernby and Avery (8); it is beset with some difficulty, however, in the case of sugar broth. With plain broth the experiment may be continued to the maximum growth possible, but in sugar broth the readings must be made before acid precipitation takes place. Slight variations in reading and in initial lag are therefore emphasized. However, the curve constructed from the results was, with one exception, smooth (Text-fig. 1). The experiment was repeated with the same general result but with discrepancies in individual tubes. Another experiment was then planned, in which in addition to the turbidity method, pH determinations were made at regular intervals in order to give an idea of the time required to reach a given pH.

TABLE VI.

Optimum Hydrogen Ion Concentration for Growth of Pneumococcus.

50 cc. portions of plain broth and 1 per cent dextrose broth were adjusted to the indicated hydrogen ion concentration and inoculated with 0.25 cc. of Pneumococcus Type I (Strain F 169). Readings were made after 6 hours at 37°C.

Initial hydrogen ion concentration of media.	Plain broth.		Dextrose broth.	
	Turbidity.	Hydrogen ion concentration.	Turbidity.	Hydrogen ion concentration.
pH	*per cent*	*pH*	*per cent*	*pH*
8.4	23	8.1	80	7.5
8.3	70	7.5		
8.0	73	7.4	100	7.2
7.8	65	7.1	37	7.5
7.5	39	7.1	39	7.1
7.3		6.9	16	7.1
7.0		6.9		6.9
6.5				6.5
6.0				6.0
5.5				5.5



TEXT-FIG. 1. Effect of initial hydrogen ion concentration on growth of pneumococcus in dextrose broth.

100 cc. portions of plain broth containing 1 per cent dextrose were adjusted to the desired pH, and 25 cc. removed for controls. After 24 hours at 37°C. as a sterility test, the 75 cc. were inoculated with 0.5 cc. of a 12 hour broth culture of Pneumococcus Type I–1 (this heavy dose was given in order to complete the experiment within 1 day). At intervals 5 cc. samples were removed for pH determinations, and when these readings indicated active growth 5 cc. samples were autoclaved for nephelometer readings. Macroscopic estimations of growth were also made to a +++ scale. The results are given in Table VII and in Text-fig. 2.

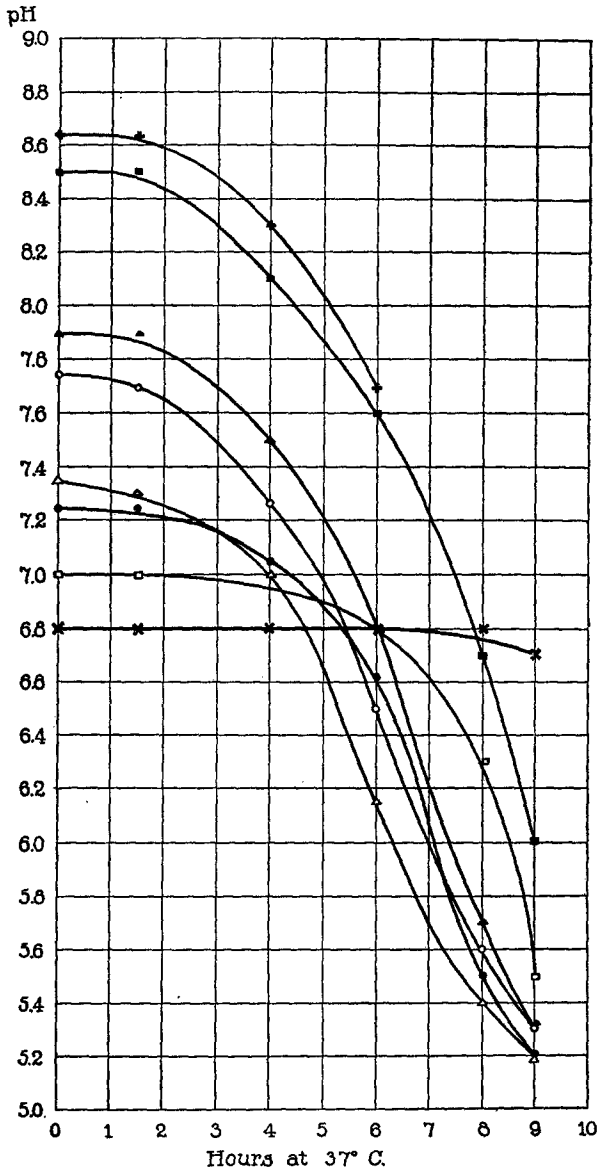
TABLE VII.

Optimum Hydrogen Ion Concentration for Growth of Pneumococcus in Dextrose Broth.

75 cc. of plain broth containing 1 per cent dextrose were inoculated with 0.5 cc. of a 12 hour broth culture of Pneumococcus Type I–1.

Hydrogen ion concentration.						Growth.		Turbidity, 6 hrs.
Control.	1½ hrs.	4 hrs.	6 hrs.	8 hrs.	9 hrs.	1 hr.	6 hrs.	
pH	*pH*	*pH*	*pH*	*pH*	*pH*			*per cent*
6.8	6.8	6.8	6.8	6.8	6.7	−	±	4.2
7.0	7.0	7.0	6.8	6.3	5.5	−	+	32.0
7.25	7.25	7.05	6.6+	5.5	5.2	+	+++	60.0
7.35	7.3	7.0	6.15	5.4	5.2	+	+++	
7.75	7.7	7.3	6.5	5.6	5.3	+	+++	82.0
7.9	7.9	7.5	6.8	5.7	5.3	−	+++	85.0
8.5	8.5	8.1	7.6	6.7	6.0	−	+±−	30.0
8.6+	8.6+	8.3	7.7			−	+	26.0

It is evident from these experiments that the optimum hydrogen ion concentration for growth of pneumococcus in dextrose broth was about pH 7.8; that is, the same as for plain broth (Text-fig. 1). Cultures grow luxuriantly between pH 7.2 and 8.6. The upper limit for growth in sugar medium is somewhat higher than in plain medium. Although fermentation in sugar broth starts at a pH of 7.8 and proceeds until a pH of 5 is reached, it is not possible to initiate growth below pH 7.0. The curve of the rate of acid production for this series has been also constructed (Text-fig. 2). At pH 7.75 to 7.3 the initial lag is decreased, but in all the tests from pH 7.2 to 8.6 after the initial



TEXT-FIG. 2. Effect of initial hydrogen ion concentration upon the rate of reaction change due to growth of pneumococcus in dextrose broth.

lag luxuriant growth occurs at about the same rate. This experiment emphasizes the fact that the term limiting hydrogen ion concentration must be carefully defined and that the final hydrogen ion concentration may be entirely different from the hydrogen ion concentration limits within which growth may be initiated.

Limiting Initial Hydrogen Ion Concentration.—It is brought out in the preceding experiments that the ordinary inoculation of pneumococcus fails to initiate growth if the hydrogen ion concentration of the medium is greater than pH 7.0, but that with growth well started at a pH above 7.0, acid production proceeds in sugar media to a final pH of 5.0. It would seem possible then that either the bacteria rapidly adapt themselves to the changing and hitherto unfavorable reaction, or that the medium itself is rendered more suitable for growth.

If the explanation lies in rapid adaptation of the organism one would expect that bacteria removed from a sugar broth at a reaction of pH 6.0, for example, could grow in fresh broth of the same reaction. An attempt to test this point was carried out as follows:

Dextrose broth was inoculated with 0.5 cc. of an 18 hour culture of Pneumococcus Type I. The change in reaction was followed, and at pH of 7.1, 6.5, 5.5, and 5.0 fresh samples of dextrose broth of varying hydrogen ion concentrations were inoculated with the actively growing culture. This broth had previously been adjusted to the desired reaction, tubed in 5 cc. portions, and incubated to test sterility. The heavy inoculation of 0.1 cc. of culture per 5 cc. of medium was used, and in addition several tubes were inoculated with the massive dose of 0.5 cc. of culture to 5 cc. of medium. The 13 hour culture (pH 5) was still viable as evidenced by the occurrence of growth when inoculated into fresh broth of pH 7.8. Only traces of disintegration of the cells were visible under the microscope. This experiment is recorded in Table VIII.

Study of Table VIII shows that it is impossible to initiate growth in media having a more acid reaction than pH 6.8 regardless of the fact that the inoculum may have been removed from an actively growing culture at ranges of acidity from pH 7.1 to 5.0. For instance, if the organisms are alive and growing at a pH 5.5, and a seeding is removed at this point and implanted in medium with a reaction of pH 6.5, no growth occurs.

TABLE VIII.

Failure of Actively Growing Cultures at pH below 6.8 to Initiate Growth in Fresh Broth of pH below 6.8.

5 cc. of dextrose broth of the indicated reaction were incubated for 24 hours at 37°C. after inoculation with cultures which had developed an acid reaction.

Culture (Type I).		1 per cent dextrose broth.	Inoculation per 5 cc.			
			0.1 cc.		0.5 cc.	
Age.	Hydrogen ion concentration.		Growth.	Final hydrogen ion concentration.	Growth.	Final hydrogen ion concentration.
hrs.	*pH*	*pH*		*pH*		*pH*
6½	7.1	7.8	++++	4.9		
8	6.5		++++	4.8		
9	5.5		++++	4.9		
13	5.0		++++	4.9		
6½	7.1	7.0	−	7.0−		
8	6.5		−	6.8+	++++	4.9
9	5.5		++++	5.0	++++	5.0
13	5.0		++++			
6½	7.1	6.8	−	6.8		
8	6.5		++++	4.9	++++	4.9
9	5.5		−	6.5	++++	4.9
13	5.0		−	6.8		
6½	7.1	6.5	−	6.5		
8	6.5		−	6.3	−	6.1
9	5.5		−	6.35	−	6.1
13	5.0		−	6.4		
6½	7.1	6.0	−	6.0		
8	6.5		−	6.0	−	5.9
9	5.5		−	5.95	−	5.8
13	5.0		−		−	5.8

Growth of Pneumococcus in Filtrates of Dextrose Broth Cultures of Pneumococcus.—An attempt was next made to determine whether dextrose broth in which pneumococcus has grown to the final hydrogen ion concentration will sustain growth if the organisms are removed by Berkefeld filtration and the reaction of the filtrate is readjusted to ranges of acidity from pH 7.8 to 6.0.

To salt-free broth 0.2 per cent sodium phosphate and 1 per cent dextrose were added, the medium was adjusted to a pH 7.8, and inoculated with Type I pneumococcus. After 24 hours incubation the culture was filtered through a Berkefeld filter. Samples of the filtrate, the pH of which was 5.2, were adjusted with sodium hydroxide to pH 6.0, 6.5, 6.8, 7.0, and 8.0; 5 cc. of filtrate required 3.3 cc. of 0.05 N sodium hydroxide to bring it to a pH 8.0. The filtrate at each pH was divided into 5 cc. portions, one of which was used as a test for sterility. Tubes containing 5 cc. of filtrate were inoculated in duplicate with 0.1 cc. of 18 hour cultures of Types I and II. The results are given in Table IX.

TABLE IX.

Growth of Pneumococcus in Filtrates of Dextrose Broth Cultures of Pneumococcus.

Dextrose broth pH 7.8. After 24 hours the culture was filtered through a Berkefeld filter. The pH of the filtrate was 5.2.

Filtrate from Type I (Strain 183) adjusted to.	48 hrs. after inoculation with.			
	Type I (Strain 183).		Type II (Strain D 39).	
	Growth.	Hydrogen ion concentration.	Growth.	Hydrogen ion concentration.
pH		*pH*		*pH*
8.0	++++	5.3		
7.0	++++	5.2	++++	5.1
6.8	++++	5.2	++++	5.2
6.5	−	6.5	−	6.5
6.0	−	6.0	−	6.0

It is evident that filtrates of dextrose broth in which pneumococcus has grown and the reaction of which has been readjusted with sodium hydroxide will not allow growth to be initiated if the readjusted pH is below 6.8.

This experiment indicates that the medium is not specifically exhausted of the substances necessary for growth, nor is there a formation of specific inhibiting bodies. This has been confirmed by growing two other strains of different types of pneumococcus in dextrose broth. After a week the cultures became sterile, the organisms were removed by centrifugation, the supernatant fluids were adjusted to

a pH 7.7, and portions of each fluid were reinoculated from fresh cultures of both organisms. Growth occurred in all portions (Table X).

TABLE X.

Dextrose Broth in Which Pneumococcus Has Grown Shows No Specific Exhaustion of Fermentable Substances and No Specific Inhibiting Substances.

1 per cent dextrose broth, pH 7.7, after 7 days incubation, broth sterile, pH 5.1. The supernatant fluid after centrifugation was adjusted with sodium hydroxide to pH 7.7, reinoculated, and incubated for 48 hours.

Supernatant fluid of broth cultures of pneumococcus.	Adjusted hydrogen ion concentration of supernatant fluid.	Reinoculation of supernatant fluid with pneumococcus.	
		Type I.	Type II.
	pH	*pH*	*pH*
Type I	7.7	5.1	5.1
" II	7.7	5.1	5.1

Exhaustion of Fermentable Substances in Plain Broth.—Since growth cannot be started in either plain broth or in dextrose broth if the hydrogen ion concentration is appreciably greater than pH 7.0 to 6.8, it is evident that this degree of acidity is unfavorable for the initiation of growth. However, pneumococcus in dextrose broth of pH above 7.0 grows to a pH of 5.0, while in plain broth of the same initial pH, growth ceases at about 7.0. Is this cessation of growth a result of the attaining of that reaction, or is it due to exhaustion of fermentable substances? To test this, plain broth cultures of pneumococcus which had developed maximum acidity, were filtered through a Berkefeld filter and the filtrate was divided into several portions and reinoculated with pneumococcus. As seen in Table XI the filtrate, both at pH 7.0 and when readjusted to its initial pH 7.5, shows no growth; on the other hand, the addition of dextrose, whether to the unadjusted filtrate, or to filtrate the reaction of which has been restored to the initial pH, is sufficient to cause abundant growth. It is evident then that the cessation of growth in plain broth when the culture has reached about pH 7.0 is due, partially at least, to exhaustion of fermentable substances. The acid produced in itself does not inhibit growth. This conclusion is emphasized by Table I in which it is shown that 0.1 per cent dextrose broth reaches a pH 6.5, 0.2 per cent dextrose broth a pH 6.0, and 0.4 per cent dextrose broth a pH 5.0.

TABLE XI.

Exhaustion of Fermentable Substances in Plain Broth by Pneumococcus.

100 cc. of plain broth, initial pH 7.5, were inoculated with Type I (Strain D 46), at 37°C. for 24 hours, and filtered through a Berkefeld filter. The filtrate, pH 7.0, was reinoculated and incubated 48 hours.

Filtrate of plain broth culture of Pneumococcus Type I.	Hydrogen ion concentration of filtrate.	Filtrate reinoculated with pneumococcus.			
		Type I.		Type II.	
		Growth.	Hydrogen ion concentration.	Growth.	Hydrogen ion concentration.
	pH		*pH*		*pH*
Unchanged	7.0	−	7.0	−	7.0
Dextrose added to 1 per cent	7.0	+++	5.0	+++	5.1
Adjusted with NaOH	7.5	−	7.5	−	7.5
" " NaOH + 1 per cent dextrose	7.5	+++	5.0	+++	5.0

DISCUSSION.

In this paper are presented facts thus far acquired in a study of acid production by pneumococcus when grown in the presence of fermentable substances. The strains of pneumococcus chosen are representative of the different serological types and in the majority of instances have been isolated from patients having lobar pneumonia. Some of these strains were freshly isolated, others have been under cultivation on artificial media for years. During this time their virulence has been maintained by animal passage and they have lost none of their biologic specificity. It is evident also from the protocols given that these conditions of preservation and animal passage have not affected the biochemical functions concerned in acid production. Moreover, it is apparent, for the limited number of strains studied at least, that no difference in the degree of acidity produced by the specific types of pneumococcus could be determined in media containing the test substances. In measuring the acidity produced by growth of pneumococcus use has been made of the colorimetric method for determining the hydrogen ion concentration. The limit of acid tolerance of pneumococcus in sugar-containing media, representing the final hydrogen ion concentration, is remarkably constant for all the strains studied.

The point of maximum acidity at which growth ceases has been defined as the final hydrogen ion concentration and has been found for all types of pneumococcus in carbohydrate media to be about pH 5. This final reaction is affected by the concentration of fermentable sugar in the medium up to 0.4 per cent. In the presence of this amount of dextrose, for instance, sufficient acid is produced during growth to bring the medium to the final hydrogen ion concentration of pH 5, but excess of sugar up to at least 4 per cent has no further influence on the final reaction. The rate of acid production, while a function of the size of the inoculum and of the optimum reaction of the medium, is, as previously shown by Chesney (9) and Cullen and Chesney (10), after the period of initial lag, rapid and constant until the final hydrogen ion concentration is reached.

In media containing 1 per cent maltose, saccharose, lactose, galactose, raffinose, dextrose, or inulin, strains of pneumococcus representing the four specific types produced acid to a final hydrogen ion concentration of pH 5. These results are in agreement with those of a previous study of 48 strains in which it was observed that these test substances are fermented by pneumococcus regardless of type differences.

Dernby and Avery have previously shown that the optimum hydrogen ion concentration for growth of pneumococcus in plain broth is pH 7.8. In the course of the present experiments these results have been confirmed and the observations extended to determine the optimum reaction for growth in media containing sugar. The initial reaction of all the media tested shows the optimum to be pH 7.8. The limits of hydrogen ion concentration within which growth can be initiated, however, were found to cover a somewhat wider range in carbohydrate-containing media (pH 8.3 to 6.8) than in similar media to which these substances are not added (pH 8.1 to 7.0). However, it is apparently impossible to initiate growth in a medium containing sugar if the initial reaction is more acid than pH 6.8, even though the organisms used for seeding are transplanted immediately from an actively growing culture at ranges of acidity varying from pH 7.1 to 5.

It has been found that in bacteria-free filtrates of plain broth cultures of pneumococcus, even when the filtrate is adjusted to the opti-

mum reaction, pneumococcus cannot be made to grow again unless small amounts of sugar (dextrose) are added. On the other hand, filtrates of dextrose broth cultures of pneumococcus, under the optimum conditions of reaction, apparently contained sufficient unutilized sugar to allow growth to occur on subsequent reinoculation. The ability of pneumococcus to grow, then, when reinoculated into the filtrate of a broth culture of pneumococcus of the same or different type, after the reaction of the filtrate has been readjusted to the optimum hydrogen ion concentration, appears to be dependent in part at least upon the presence of a residuum of fermentable substance left unmetabolized by previous growth. It is probable, however, that the exhaustion of fermentable carbohydrate from culture media is only one of many factors involved in the complex phenomenon of growth inhibition.

SUMMARY.

1. The optimum hydrogen ion concentration for growth of pneumococcus is pH 7.8.

2. In broth cultures growth of pneumococcus continues until a final hydrogen ion concentration of about pH 5.0 is reached, if sufficient fermentable carbohydrate (above 0.4 per cent) is present. Apparently this acidity is sufficient in itself to stop growth.

3. If less carbohydrate is present in the medium growth ceases at a lower hydrogen ion concentration, apparently because of exhaustion of carbohydrate. If no carbohydrate is present save that extracted from the meat of which the broth is made (plain broth medium), growth initiated at pH 7.8 (optimum reaction) ceases at about pH 7.0.

4. If bacteria-free filtrates of plain broth cultures in which growth has ceased are readjusted to pH 7.8 and reinoculated with pneumococcus, no growth occurs unless carbohydrate is added. However, if bacteria-free filtrates of dextrose broth cultures in which growth has ceased (pH 5) are readjusted to pH 7.8 and reinoculated with pneumococcus growth occurs.

5. Cultures of pneumococcus with all the carbohydrates which were fermentable under the conditions used, namely maltose, saccharose, lactose, galactose, raffinose, dextrose, and inulin, gave identical results in the rate of reaction change, and final hydrogen ion concentration (pH 5.0) attained.

6. The different immunological types of pneumococcus, for the limited number of strains studied, behaved alike in fermenting the carbohydrates mentioned above.

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