THE RELATION OF HYDROGEN-ION CONCENTRATION TO THE GROWTH, VIABILITY, AND FERMENTATIVE ACTIVITY OF STREPTOCOCCUS HEMOLYTICUS

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I. THE FINAL HYDROGEN-ION CONCENTRATION PRODUCED BY STREPTOCOCCUS HEMOLYTICUS IN BROTH CONTAINING VARIOUS FERMENTABLE SUBSTANCES

In 1912, Michaelis and Marcora (1912) working with a culture of *Bact. coli* in lactose broth were able to show by means of accurate electrometric measurements, that this organism carries its fermentation of the sugar to a definite level of hydrogen-ion concentration and then ceases its activity. This point is reached regardless of the initial reaction of the medium and can be described as a physiological constant for the particular organism used. This finding was confirmed three years later by W. M. Clark (1915b)[1] who pointed out that the final hydrogen-ion concentration established as a physiological constant by Michaelis and Marcora for a single strain of *Bact. coli* applied to other strains as well. That the hydrogen-ion concentration of the culture, rather than the total acid produced, is the factor limiting activity of the organism seemed evident from the work of Clark. The usefulness of this so-called physiological constant appeared later as the result of the researches of Clark and Lubs (1915) who suggested a method of differentiating the bacteria of the colon-aerogenes group by means of a correlation with gas formation of the final hydrogen-ion concentration produced in glucose broth. In this work was laid the experimental foundation of the methyl

[1] Bibliography is found at the end of the third article in this series, p. 231.

red test in use at the present time. Ayers (1916) in an investigation of the final hydrogen-ion concentration in some 200 cultures of streptococci was able to demonstrate a somewhat higher acidity[2] in cultures of the non-pathogenic than in those of pathogenic species grown upon glucose broth. Later work by Ayers, Johnson, and Davis (1918), as well as by Avery and Cullen (1919a), has led to the suggestion of a rapid presumptive test for the differentiation of bovine and human streptococci based upon differences in the final hydrogen-ion concentration produced in glucose broth. However, as Brown (1920) has pointed out, no single procedure can perhaps serve to differentiate the two varieties absolutely inasmuch as atypical strains are somewhat frequent. Cullen and Chesney (1918), Jones (1920, 1920a), Avery and Cullen (1919b), and Lord and Nye (1919), working with pneumococci of the various types in glucose broth, have found a final hydrogen-ion concentration that is in close agreement with the constant established for the streptococci. This value appears to be the same in all types irrespective of immunological character. The works of Fred and Loomis (1917) upon alfalfa bacteria, of Bunker (1916–1917, 1919) and Davis (1918) upon *Corynebact. diphtheriae*, of Itano (1916a, 1916b) upon *B. subtilis* and certain streptococci, of Cole and Onslow (1916) upon the typhoid group, of Clark (1917) upon *Lactobacillus bulgaricus*, of Waksman and Joffe (1920) upon Actinomycetes, of Ayers and Rupp (1918) upon members of the alkali-forming group, of Wolf and Harris (1917a, 1917b) upon *Clostridium welchii* and *C. sporogenes*, of Gillespie (1916, 1918) on soil organisms, and of Cohen and Clark (1918) upon various organisms are indicative of an attempt on the part of present-day workers to gain a more accurate knowledge of the metabolic activities of micro-organisms through the measurement of changes in the hydrogen-ion concentration brought about in culture media. Determinations of titratable acidity and ammonia, according to Kligler (1916), give an indication of the extent of carbohydrate

[2] The terms *acid* and *acidity* in the present paper refer to true acidity as expressed in terms of P_H, except when the reference is specifically to *titratable acid* or *acidity*.

and protein splitting by bacteria, whereas measurement of the hydrogen-ion concentration in cultures measures the resultant of both these actions.

It seemed advisable at the beginning of the present phase of the investigation to obtain information as to the level of final acidity produced by *Streptococcus hemolyticus* in broth media containing a number of the common fermentable substances employed in the bacteriological laboratory. Accordingly experiment I was carried out.

Methods and technic

Culture. All of the work to be described in the present paper was carried out on one pure strain of *Streptococcus hemolyticus.* This strain, designated as the "H," was originally isolated from the lung in a fatal case of bronchopneumonia complicated by endocarditis, and corresponds to culture number 136 in the series obtained during the investigation of pneumonia in military camps by the Rockefeller Commission. The "H" strain was of high virulence, owing to repeated passage through rabbits in an investigation of experimental streptococcus empyema, and the pleural fluids of such animals, taken at autopsy with sterile precautions (Gay and Stone, 1920), were found to serve as an excellent source of culture material. All pleural fluids were stored in the ice chest as the contained organisms have been found to remain viable under such conditions for a number of weeks. A transplant of 0.2 cc. of the pleuritic exudate was made into 5 cc. of 1 per cent glucose broth and the tube incubated for eighteen hours. As this first generation culture invariably contained a considerable amount of cellular débris, a second subculture was prepared in a similar manner. This second generation served as a source of inoculum in practically all of the experiments to be described. The eighteen-hour incubation period was chosen inasmuch as preliminary tests had shown that rapid growth nearly always obtained in sub-cultures prepared from a parent culture of this age.

Culture media. Beef infusion broth served as the basis of the media employed throughout the work as it is a generally

recognized fact that the pathogenic streptococci develop more luxuriant growth upon this medium than upon broth prepared from beef extract. In some of the experiments "Bacto-beef" (Digestive Ferments Company) was employed instead of beef juice as a base. Growth upon this medium was found to be as luxuriant as upon the usual beef infusion broth. The broth contained 1 per cent peptone (Difco or Parke, Davis, and Company), and 0.5 per cent NaCl. Adjustment to the desired P_H was made according to the method previously described. The limits of P_H, 7.0–7.6, were found to favor luxuriant growth of the organism. The prepared broth was always incubated for twenty-four hours previous to inoculation to insure its sterility.

P_H determinations.[3] These were made by the method described in a former paper using 1 cc. of culture plus 4 cc. of freshly boiled and cooled distilled water. A tube containing the same materials without indicator was always used by the method of superposition to eliminate as far as possible factors of color and turbidity. Determinations carried out in this way permitted readings to within 0.05 P_H in nearly all cases.

Experiment I. The final hydrogen-ion concentration of Streptococcus hemolyticus in broth containing various fermentable substances commonly employed in the bacteriological laboratory; also an attempt to investigate the possibility of an experimental adaptation to a given sugar medium, through repeated transplantation.

Inoculation of 0.4 cc. of an eighteen-hour, second-generation culture was made into 10 cc. lots of beef infusion broth containing the given fermentable material in 1 per cent concentration. Transplants from each tube were made, into sterile lots of media of corresponding composition after twenty-four hours incubation. In this manner five generations were carried. Although the "H" strain had previously been found to produce the characteristic final hydrogen-ion concentration quite con-

[3] The symbol P_H of Sörensen is used throughout to designate the hydrogen ion concentration.

sistently within the first twenty-four hours following incubation, nevertheless in this experiment it was decided to allow a forty-eight-hour incubation period before making P_H determinations to insure the completion of the fermentation.

Reference to table 1 shows that of the several groups of substances tried only the hexoses and disaccharides were fermented by the streptococcus. A wide variation in final P_H is noted. No explanation of these differences is attempted at the present time. Clark (1915b) working with *Bact. coli*, reports lower P_H levels in glucose broth than in lactose broth, while Jones (1920) has described a similar phenomenon in cultures of *Streptococcus hemolyticus* and pneumococci. Similar results are evident in the present experiment. An interesting fact brought out is that plain broth shows an increase in hydrogen-ion concentration. It is also to be noted that in no case in which fermentation did occur was the characteristic final P_H reached in the first generation. This would seem to indicate that in procedures for differentiation based upon final P_H levels, several transfers of the cultures should be made upon the same medium before conclusions as to the final hydrogen-ion concentration are drawn. In nearly all cases the characteristic final value was reached after one transfer.

The fact that plain broth shows an increase in hydrogen-ion concentration when inoculated with the streptococcus would seem to indicate that sufficient muscle sugar is present to permit fermentation to the P_H level indicated. To decide this point, a lot of infusion broth was inoculated with *Bact. coli* to ferment out any free sugar, after which it was filtered, adjusted, and sterilized. Upon inoculation with a culture of *Streptococcus hemolyticus* it was found that the final P_H was the same as that noted in the experiment just described. In this case the initial P_H of the broth was slightly lower, namely, 7.35. A similar result was experienced when sugar-free, bacto-beef broth was tried. In their studies of the metabolism of *Streptococcus pyogenes* and other organisms Kendall and his associates (1912c, 1912a) found increases in titratable acidity in plain broth cultures but carried out no determinations of hydrogen-ion concen-

tration. According to these investigators, the phenomenon may be explained on the basis of a selective action of the organism in question upon that portion of Witte's peptone which Pick (1898) has shown contains a relatively large fraction of a sub-

TABLE 1

Experiment I

NUMBER	CARBOHYDRATE	P_H (INITIAL)	GENERATION				
			1	2	3	4	5
1	None	7.50	+++ 6.70	++ 6.70	++ 6.70	++ 6.70	
2	Glucose	7.50			+++ 5.10	+++ 4.80	+++ 4.85
3	Fructose	7.50	+++ 5.30	+++ 5.10	+++ 5.10	+++ 5.10	+++ 5.05
4	Mannose	7.50	+++ 5.40	+++ 5.20	+++ 5.25	+++ 5.20	+++ 5.20
5	Galactose	7.50	+++ 5.50	+++ 5.40	+++ 5.40	+++ 5.40	+++ 5.30
6	Xylose	7.50	++ 6.60	++ 6.70	++ 6.70	++ 6.70	
7	Sucrose	7.50	+++ 5.35	+++ 5.20	+++ 5.10	+++ 5.15	+++ 5.10
8	Lactose	7.50	+++ 5.50	+++ 5.50	+++ 5.40	+++ 5.40	+++ 5.40
9	Maltose	7.50	+++ 5.40	+++ 5.30	+++ 5.15	+++ 5.10	+++ 5.15
10	Inulin	7.50	++ 6.60	++ 6.60	++ 6.70	++ 6.70	++ 6.70
11	Glycerol	7.50	++ 6.60	++ 6.70	++ 6.70	++ 6.70	++ 6.50
12	Mannite	7.50	++ 6.60	++ 6.60	++ 6.70	++ 6.70	++ 6.70

stance reacting typically like a carbohydrate. Although the peptone used in the present experiments was not Witte's it seems entirely possible that American peptones such as the one used here (Parke, Davis and Company) might contain a similar carbohydrate substance. The fact that a definite increase in hydrogen-ion concentration has always been observed in the sugar-free broth employed surely would lend support to such a supposition.

II. THE INFLUENCE OF VARYING AMOUNTS OF GLUCOSE AND BUFFER SALTS UPON THE FINAL HYDROGEN-ION CONCENTRATION OF STREPTOCOCCUS HEMOLYTICUS

It has long been recognized that the acidity produced by certain organisms in culture media results from the elaboration of acid substances through a fermentation of material, mainly of carbohydrate nature. With the introduction of accurate methods of evaluating the acidity produced in bacterial fermentations through a determination of the concentration of the hydrogen-ions, it became necessary to investigate the factors which may be operative in the production of a limiting or final hydrogen-ion concentration. Thus, Clark and Lubs (1915) in their work on the differentiation of the bacteria of the colon-aerogenes family, used media containing amounts of glucose varying from 0 to 0.5 per cent and demonstrated that by increasing the concentration of the sugar up to a certain point a greater final acidity resulted. If sufficient sugar was present for the limiting acidity to be produced, no alkaline reversion occurred in their cultures. Browne (1914), using cultures of *Bact. coli* in lactose-broth, found that acid production was less marked in media containing under 1 per cent sugar but that the use of amounts over 1 per cent resulted in no increase. Browne titrated his cultures with N/20 NaOH but failed to make determinations of the final hydrogen-ion concentration. Avery and Cullen (1919b) found that pneumococci were able to reduce the P_H of glucose-broth from 7.50 to 5.10 provided 0.4 per cent of the sugar was present. Increasing concentrations of glucose up to 4 per cent showed no change in

final P_H. In the work of the same investigators (1919a) upon Streptococci of human and bovine origin it was shown that the same final P_H is reached in broth containing 0.5, 1, or 1.5 per cent of glucose. Sekiguchi (1917) found the highest production of acid by streptococci with 0.5 to 2 per cent of glucose. Amounts of sugar over 5 per cent caused reduction in acid formation though growth was not hindered. H. Jones (1920) has recently found that a number of organisms are able to produce their characteristic final hydrogen-ion concentration provided 0.2 per cent or more glucose be present in the medium. He failed to state the initial P_H of the medium which factor has an important bearing on the minimum concentration of a sugar needed for production of the final acidity by any given organism. The effect of varying amounts of xylose upon the production of volatile acid by xylose fermenting organisms has been studied by Fred, Peterson, and Davenport (1919) who found that 2 per cent of the sugar gave the maximum production of acid. The presence in the culture medium of substances which through their buffer effect have the power of neutralizing some of the acid as it is produced is of interest and importance in this connection.

Henderson and Webster (1907) in 1907 suggested the use of phosphates to preserve neutrality in media during the growth of acid- or alkali-forming organisms, and Clark (1915a) has more recently pointed out in considerable detail the great importance of properly buffered media in bacteriological work. Using lots of broth containing different buffers, Clark (1915b) showed that *Bact. coli* produces somewhat lower levels of P_H in the more highly buffered media.

Kligler (1916) working with cultures of *Bact. cloacae*, *Bact. aerogenes*, and *Bact. coli* studied the final P_H as influenced by different concentrations of peptone, Na_2HPO_4, and glucose. The concentration of peptone was found to influence the utilization of glucose by the organisms in such a way as to result in a lower final P_H with a low peptone concentration in the medium. In some cases the presence of buffer allowed all of the sugar to be used up with a subsequent rise of P_H thus indicating that an

alkaline phase had been initiated through the splitting of peptone. The presence of buffer, according to Kligler, keeps the hydrogen-ion concentration below the lethal point and thus allows the organism to continue its activity over a longer period. As a result of this regulatory power the amount of glucose which may be used will vary, within limits, with the relative amount of buffer material present. Bronfenbrenner and Schlesinger (1918) working with *Bact. coli* have tried similar experiments by noting the effects of varying amounts of lactose, peptone, and buffer salts upon gas formation and final P_H. After trying some 294 combinations, these investigators concluded that with any given concentration of carbohydrate the amount of free acid depends upon the concentration of buffer in the medium. As the amount of peptone increases, the per cent of sugar attacked is smaller and lower hydrogen-ion concentrations result. The necessity of carefully controlling the composition of media employed in fermentation experiments is emphasized.

From the foregoing review the following facts seem to have been well established:

1. In any given medium a definite concentration of sugar must be present if the organism in question is to produce its characteristic final hydrogen-ion concentration.

2. This minimum concentration of sugar will depend upon the concentration of buffer salts present, as well as upon the concentration of peptone in the medium.

3. In making estimations of this minimum concentration of sugar required for the production of the final hydrogen-ion concentration the quantity of buffer should be known as well as the initial P_H of the culture medium.

4. With increasing concentrations of buffer salts there is an increased neutralizing power which delays the production of the final acidity level, thus allowing the organism more time for fermentation.

Experiment II. The effect of varying concentrations of glucose upon the final hydrogen-ion concentration of Streptococcus hemolyticus

Ten cubic centimeter amounts of beef infusion broth containing concentrations of glucose varying from 0.10 to 1 per cent were inoculated with 0.4 cc. of an eighteen-hour culture of *Streptococcus hemolyticus* in 1 per cent glucose broth and incubated for three days to insure the completion of the fermentation. P_H readings were then made. The results are shown in table 2.

TABLE 2
Experiment II

NUMBER	MEDIUM	P_H (INITIAL)	P_H (FINAL) IN GLUCOSE (PER CENT)					
			0	0.1	0.2	0.3	0.5	1.0
1	Beef infusion broth	6.90		5.60	5.00	5.10	5.05	5.10
2	Beef infusion broth (sugar free)	7.35	6.70	6.05	5.60	5.10	5.15	5.00

In (1) which was adjusted to an initial P_H of 6.9 the final P_H was attained in a glucose concentration of 0.2 per cent, whereas in (2) which was adjusted to an initial P_H of 7.35 the final value was not shown in the 0.2 per cent glucose but did appear in the 0.3 per cent tube. As would be expected the minimum concentration of glucose needed to give the characteristic final P_H is dependent upon the initial P_H of the broth. Amounts of glucose over this minimum concentration have no further effect upon the level of the final hydrogen-ion concentration.

Experiment III. The influence of a buffer salt upon the final hydrogen-ion concentration of Streptococcus hemolyticus in broth containing varying concentrations of glucose

Bacto beef broth was adjusted and distributed in twelve lots in flasks. After autoclaving, the requisite amounts of glucose and di-potassium phosphate, K_2HPO_4, were added in the form of sterile 10 per cent solutions bringing the total volume of material in each flask to 25 cc. Following twenty-four

hours incubation to insure sterility each flask was inoculated with 1.25 cc. of an active twenty-two-hour culture. Determinations of P_H and "reaction" were made after an incubation period of four days. The "reaction" was determined by titrating 5 cc. of culture with N/50 NaOH, using neutral red as an indicator and calculating the number of cubic centimeters of N/1 NaOH needed to neutralize the acid in 100 cc. of culture. Table 3 contains the results of the experiment.

TABLE 3

Experiment III

NUMBER	GLUCOSE	K_2HPO_4	P_H (INITIAL)	P_H (FINAL)	"REACTION"*
	per cent	*per cent*			
1	0.3	0	6.90	5.10	0.72
2	0.3	0.2	6.90	5.05	1.41
3	0.3	0.5	7.20	5.20	2.13
4	0.3	1.0	7.20	6.30	2.43
5	0.5	0	6.75	5.20	0.70
6	0.5	0.2	6.70	5.00	1.54
7	0.5	0.5	7.20	5.05	2.18
8	0.5	1.0	7.20	6.20	2.16
9	1.0	0	6.90	5.15	0.81
10	1.0	0.2	6.90	5.00	1.56
11	1.0	0.5	7.20	4.90	3.36
12	1.0	1.0	7.20	5.20	4.73

* Cubic centimeters of N/1 NaOH required to neutralize 100 cc. of culture.

As will be seen by referring to table 3 the final P_H characteristic of the streptococcus is not reached in the media containing 0.3 per cent and 0.5 per cent glucose plus 1 per cent phosphate (numbers 4 and 8 in table). These concentrations of glucose are apparently not sufficiently great to allow the formation of enough acid to bring the culture to the characteristic level, whereas in the case of the 1 per cent glucose plus 1 per cent phosphate a characteristic final P_H is reached. Correlated with these facts are the differences in titratable acid as shown in the last column of the above table. It is an interesting fact that virtually the same final P_H is shown in the greater number of the

above cases and yet the total quantities of actual acid, as shown by titration, are widely different. No better illustration of the efficiency of a buffer could be offered. Very obviously the utilization of glucose is here closely related to the concentration of buffer present. A further fact, of interest and importance, is that the final hydrogen-ion concentration rather than the total acid produced is the factor which limits the fermentative activities of the organism.

Experiment IV. The influence of horse serum in glucose broth upon the final P_H *of Streptococcus hemolyticus*

Ten cubic centimeter lots of beef infusion broth (sugar-free) containing varying amounts of glucose and horse serum were prepared and inoculated with 0.4 cc. of an eighteen-hour culture. After an incubation of three days P_H determinations were made. The results of (2) in experiment II are inserted in table 4 for purposes of comparison.

TABLE 4

Experiment IV

NUMBER	HORSE-SERUM	P_H (INITIAL)	P_H (FINAL) IN GLUCOSE (PER CENT)					
			0	0.1	0.2	0.3	0.5	1.0
	per cent							
1	5.0	7.40	6.80	6.70	6.10	5.05	5.00	5.00
2	10.0	7.60	6.80	6.60	5.90	5.15	5.00	5.10
2 (exp. II)	None	7.35	6.70	6.05	5.60	5.10	5.15	5.00

As in experiment II it is to be noted that 0.3 per cent glucose is the minimum concentration which will permit the attainment of the characteristic final P_H. The greatest differences in P_H between the media containing horse serum and (2) of experiment II are seen in the tubes containing 0.1 per cent and 0.2 per cent glucose. It seems possible that in these cases the horse serum prevents the increase in acidity of the medium to a small extent through its action as a buffer. In those tubes containing sufficient glucose for the production of the final P_H characteristic of the organism no differences in the level of this final value are seen. That we do have a decided difference in the rates of acid production will be shown in a later experiment.

Experiment V. The buffer action of horse serum in broth

To investigate further the buffer effect of horse serum titration curves of broth containing 1 per cent glucose, 1 per cent glucose plus 5 per cent horse serum, and 1 per cent glucose plus

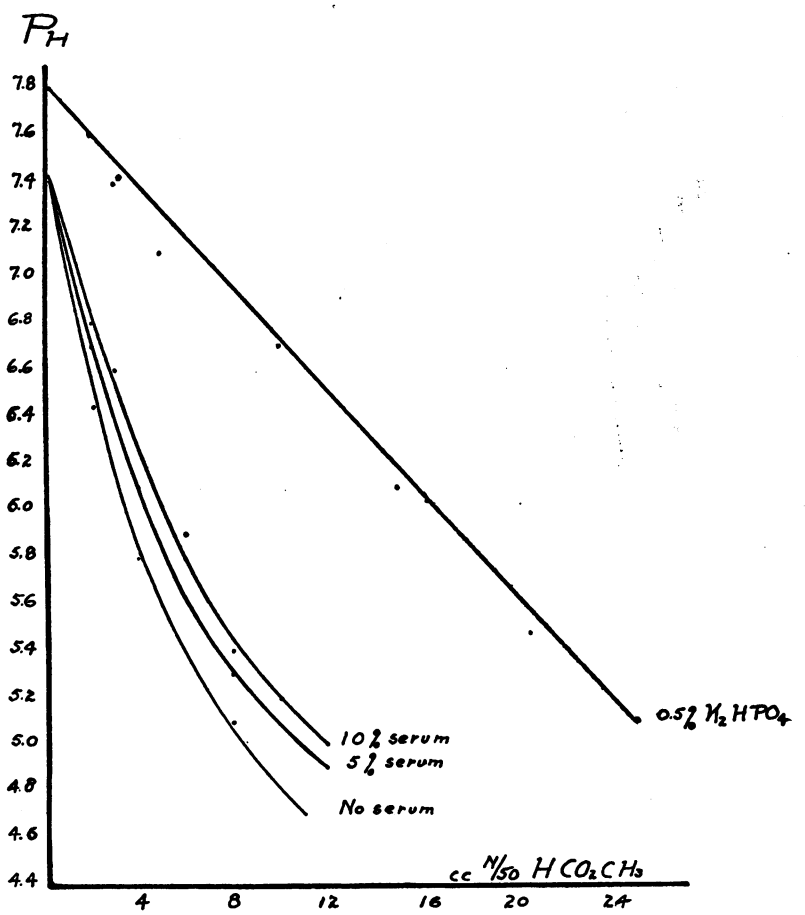

FIG. 1. EXPERIMENT V

10 per cent horse serum were plotted after the following procedure had been carried out: To 10 cc. portions of the three types of broth mentioned above, amounts of N/50 acetic acid varying from 1 to 12 cc. were added and the P_H taken. The curves were

plotted using the cubic centimeter of acid as abscissae and the P_H readings as ordinates. Reference to the curves (fig. 1) will show that horse serum in these concentrations exerts a slight but distinct buffer effect. The 10 per cent series does not show a much greater buffer action than the 5 per cent series however, and the effect in no case is anything like that noted in the case of K_2HPO_4 (experiment III).

III. THE RATE OF ACIDITY FORMATION IN CULTURES OF STREPTOCOCCUS HEMOLYTICUS

Considerable work by a number of investigators has demonstrated that the life cycle of a given organism, as measured by the number of viable cells present at various intervals following inoculation, may be separated into very definite periods. Thus, Chesney (1916) has suggested a division into four phases: (1) latent period or lag, (2) maximum growth period, (3) stationary period, (4) period of decline.

No sharp dividing lines may be drawn between the periods, and their duration will vary in the case of the same organism with such factors as the amount of inoculum, age of parent culture, and initial reaction of the medium. Buchanan (1918) described seven periods in the life of an organism: (1) initial stationary phase; (2) lag phase when growth proceeds at a slowly accelerating rate; (3) maximum or logarithmic period in which the rate of increase in numbers is constant; (4) period of negative growth acceleration, the organisms are increasing at a decreasing rate; (5) maximum stationary period; no increase in numbers; (6) period of accelerated death, decrease in taking place at an increasing rate; (7) logarithmic death phase; death is occurring at a constant rate.

With the development of procedures for the mathematical analysis of the several phases (Buchner, Longard, and Riedlin (1887), Buchanan (1918), Slator (1917), Ledingham and Penfold (1914) has come the possibility of more definite knowledge concerning the growth activities of organisms.

A search through the literature reveals the fact that the latent period or lag phase has received the bulk of the attention of workers in this field. Müller (1896) perhaps was the first to recognize the phenomenon while working with cultures of *Bact. typhosum* at temperatures simulating febrile conditions. The duration of lag was found by him to vary with the age of the culture used for seeding, being shorter for young than for older cultures. He believed the phenomenon to be the result of an alteration of the cells sustained upon transplantation to a new medium, the duration of lag representing the time required for the organisms to recover from the injury. Rahn (1906), working with *Ps. fluorescens*, studied the influence upon lag of the amount of inoculum and concluded that the larger the number of organisms used for seeding, the shorter the lag. Penfold (1914) later demonstrated that this effect held, up to a certain limit, beyond which an increase in the amount of inoculum exerted no influence upon the duration of the lag period. In case of small inocula, however, Penfold showed that a diminution in amount of seed invariably caused a lengthening of lag. He found that older cultures caused lengthening of lag only up to a certain point, for example, a four-day culture gave the same duration of lag as a twelve-day culture in the case of *Bact. coli*. Barber (1908) working with single cells (*Bact. coli*) was the first to show that under proper conditions lag may be eliminated. He used rapidly dividing cells which were accustomed to the medium employed and was able to find no evidence of inhibition upon transplantation. This observation has received substantiation at the hands of Penfold (1914), Chesney (1916), and Salter (1919), all of whom worked with *Bact. coli*. Coplans (1909) also states that with *Bact. coli*, there is ordinarily no absolute lag upon transplantation to a favorable medium. New milk ordinarily possesses inhibitory properties but this investigator found that heating momentarily to 100°C. caused a disappearance of this special inhibitory quality. Salter (1919) found also that the age of the parent culture exerted a considerable influence upon the duration of lag, thus confirming the observations of previous investigators. Lane-Claypon (1909) has studied the

rate of growth of organisms as affected by different temperatures and has demonstrated a conformity of her curves with the Van't Hoff-Arrhenius law within certain limits.

The various other phases in the life of a culture have been investigated to a less extent but from the work of Buchanan (1918) and Ledingham and Penfold (1914) it seems probable that growth is a discontinuous process in the sense that development of a given organism is dependent upon different laws in the successive phases of the life of the culture.

An illustrative curve follows:

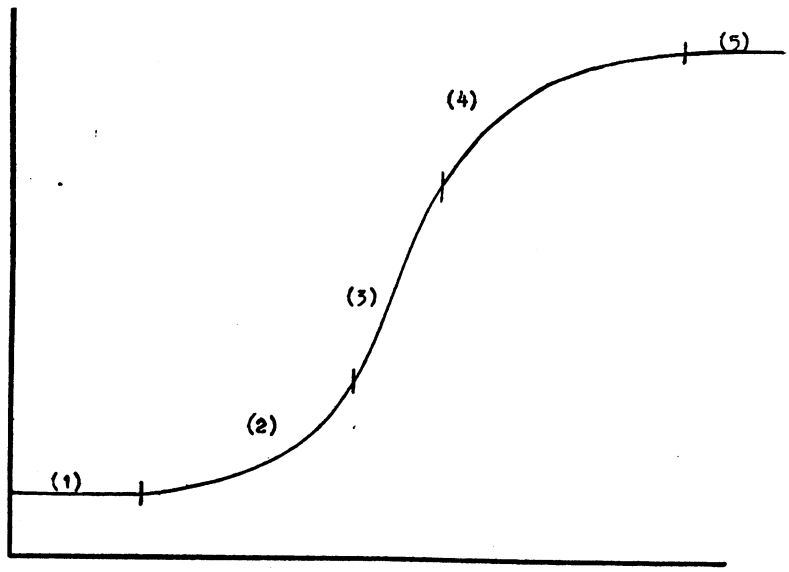

FIG. 2. ILLUSTRATIVE CURVE OF ACID FORMATION BY STREPTOCOCCUS HEMOLYTICUS IN GLUCOSE BROTH

(1) Initial stationary period.

(2) Lag period. Acid formed at a slowly increasing rate.

(3) Maximum period. Acid formed at a constant, maximum rate; curve is an oblique, straight line.

(4) Period of negative acceleration. Acid formed at a decreasing rate.

(5) Maximum stationary period. Final P_H has been attained; curve is a straight line parallel to the abscissa.

If broth containing glucose be inoculated with an actively growing culture of *Streptococcus hemolyticus* and incubated, there ensue changes in the hydrogen-ion concentration of the medium culminating in the establishment of a limiting or final reaction. A study of these changes, as measured at regular intervals, indicates that the course is a perfectly definite one capable of being separated into the following characteristic phases: (1) Initial stationary period, no change in reaction; (2) latent or lag period, acid formation at a slowly increasing rate; (3) maximum period, acid formation at a constant rate; curve an oblique straight line; (4) period of negative acceleration, acid formation at a decreasing rate; (5) maximum stationary period, final P_H reached, curve a straight line parallel to the abscissa.

It will be noted that this sub-division of the course of reaction change corresponds with Buchanan's life phases of a bacterial culture based upon numerical determinations of viable organisms, with the exception that his two final periods, representing a decrease in number, cannot, of necessity, apply to an acid curve such as is characteristic for the streptococcus. The work of Cullen and Chesney (1918) on pneumococci has shown a close relationship between growth-rate and speed of acid production in plain broth, and accordingly these observers have concluded that acid formation is to be considered as an active metabolic process, closely associated with the growth activities of the organism. In examining the curves of Cullen and Chesney one is struck by the close parallelism that exists between the various phases in the life of the pneumococcus, as measured by numbers of viable cells on the one hand, and by acid formation on the other hand. As might be expected, a rise in the growth curve always preceded slightly a rise in acidity. Lord and Nye (1919) have reported results of similar nature on pneumococci grown in glucose broth. During the first 12 hours of their experiment the medium was found to change in reaction from P_H 7.65 to 5.25. Up to this point, a rapid increase in the number of cells was evident, but during the subsequent acidification to the final P_H, 5.15, a rapid decrease in viable organisms was apparent.

In both of these investigations it is evident that the maximum changes in acid formation take place simultaneously with a rapid development and multiplication of the bacteria and thus show a conformity with the conception of Slator (1916) that "Chemical reactions brought about by microorganisms usually proceed under conditions where development of the organism and changes in the composition of the nutrient medium take place simultaneously." H. M. Jones (1920a) however, has recently obtained results which contradict the work of Cullen and Chesney, and Lord and Nye. Using cultures of pneumococci in glucose broth this investigator has shown that the growth curve rises sharply at the fourth to fifth hour while the onset of the maximum period of acid formation is delayed until the twelfth hour. Examination of the curves of this experiment shows the maximum period of growth to be associated with but a slight alteration in the reaction of the medium (7.4–7.0), whereas, the interval of acid formation at a maximum rate corresponds with the period of growth at a decreasing rate. This finding corresponds more or less closely to the observations of Cohen and Clark (1918) upon *Bact. coli* in glucose broth cultures. The growth curve was found to rise five hours previous to the onset of the maximum period of acid production, and, as in the experiments of Jones, the maximum period of acid formation was found to be coincident with the period of growth at a decreasing rate. At the point where strong symptoms of growth inhibition appeared, the P_H was found to correspond to the region at which acetic acid had been previously shown to check growth (5.5–5.7). The fermentative activity, however, was not seriously checked until the culture approached the region in which HCl had been found to inhibit growth (4.6–5.0). From a consideration of these findings it will appear, in the cases of *Bact. coli* and the pneumococcus at least, that the hydrogen-ion concentration may exert independent effects upon growth, on the one hand, and upon acid formation on the other, so that in experiments designed to follow the acid production of organisms in carbohydrate media it will be unsafe to assume that maximum changes in reaction parallel maximum rates of multiplication of bacterial cells.

Clark (1915b), working with *Bact. coli*, was perhaps the first to follow reaction changes in bacterial cultures by means of determinations of hydrogen-ion concentration. No change in P_H was noted under a period of ten hours in his experiment. Itano (1916a) followed the changes in acidity in cultures of *B. subtilis* and noted in certain media of unfavorable initial P_H that an "automatic adjustment" toward a more favorable reaction occurred during incubation. Working with *Clostridium perfringens* (*C. welchii*) and *C. sporogenes* (Metchnikoff), Wolf and Harris (1917b) found that curves of acidity change followed closely those of amino acid formation and gas production. Avery and Cullen (1919b) used media of different initial P_H with pneumococci and demonstrated that after completion of lag, growth, as evidenced by the rate of reaction change, proceeded at about equal speeds. Neither the final P_H nor the rate of acid formation was affected by the use of various available mono- or di-saccharides. The maximum period was found to lie between the fourth and eighth hours following seeding. Bunker (1919) noted an initial acidity rise followed by alkaline reversion in cultures of *Corynebact. diphtheriae* and apparently has shown that toxin production is closely associated with this phenomenon, as no toxin could be demonstrated in cultures which failed to exhibit an alkaline reversion. In a study of the logarithmic or maximum period in cultures of several organisms by Cohen and Clark (1918) it was observed that bacteria may multiply rapidly for a time in media varying considerably in initial reaction. The maximum period of growth in the case of *Bact. coli* fell between the fifth and tenth hours. Schoenholz and Meyer (1919), in their work on *Bact. typhosum*, have reported changes in the growth curve through the influence of hydrogen-ion concentration. Thus they found that growth at a maximum rate set in five hours following incubation, if the initial P_H of the medium was 7.0. At lower and higher levels lag was of longer duration.

Avery and Cullen (1919a), using streptococci of human and bovine origin, found the greatest increase in acidity between the seventh and twelfth hours, using eighteen-hour cultures as

sources of inocula. H. Jones (1920) has recently observed that in the case of pathogenic streptococci the age of the parent culture employed may exert a considerable influence upon the abundance of growth in sub-cultures which may, in turn, be reflected in the final P_H values. He also observed that cultures which were placed under conditions which tended to delay growth failed to show the characteristic final P_H. The statement frequently made that the final P_H of an organism is eventually reached, provided the culture exhibits growth, obviously can not apply to a delicate organism such as the streptococcus. Thro (1915) called attention to the same fact in his observation that with streptococci variations in luxuriance of growth were associated with differences in the quantities of acid substances produced.

Slator (1916) has devised an ingenious method for measuring the rate of growth of a lactic acid-forming organism through an indirect application of the titration values obtained at definite intervals throughout the course of the experiment. Using the formula suggested in a previous work (1917) he was able to show close agreement in the values of the constant, k, in different determinations. The possibility of simultaneous acid and alkaline fermentations in cultures of certain organisms has been emphasized by Ayers and Rupp (1918) who state that such actions may complicate and decrease the value of acidity determinations in certain cases. Methods of measuring both fermentations have been suggested by these investigators.

From the foregoing review it would appear that a study of the progress of reaction changes in cultures of *Streptococcus hemolyticus*, in order to furnish data of value, must of necessity entail an investigation of a number of interacting factors. Accordingly, experiments were planned to study the rate of acid formation as influenced by the following: (1) Amount of inoculum; (2) age of parent culture; (3) presence of a body fluid, horse serum, (4) initial reaction of medium.

Experiment VI. The influence of the amount of inoculum upon the rate of acid formation in glucose broth

Twenty cubic centimeters of 1 per cent glucose broth, P_H 7.10, were inoculated with varying amounts of an active, eighteen hour culture of *Streptococcus hemolyticus* in 1 per cent glucose broth and incubated. At two-hour intervals P_H determinations were made on 1 cc. samples removed from the cultures with aseptic precautions. All cultures remained uncontaminated throughout the entire period of the experiment. The results of the experiment are to be found in table 5 and figure 3.

TABLE 5

(*Experiment VI*)

NUMBER	INOCULUM	DURATION STATIONARY PERIOD	DURATION LAG PERIOD	ONSET OF MAXIMUM PERIOD	DURATION MAXIMUM PERIOD	P_H LOWERING (MAXIMUM PERIOD)	
						Total	Per hour
	cc.	*hours*	*hours*				
1	0.2	8	6+				
2	0.4	2	8	10	2	0.8	0.4
3	0.8	None	8	8	4	1.35	0.34
4	2.0	None	6	6	2	1.40	0.70
5	4.0	None	4	4	6	1.45	0.24

Reference to the curves (fig. 3) shows that the rates of acid formation are at least roughly proportional to the quantities of inoculum used. It is interesting to find that the hourly rate (table 5) during the maximum period is least in the case of (5) notwithstanding the fact that this contained the largest inoculum. In other words, cultures (4), (3), and (2) though showing more prolonged lag periods than (5), are able to proceed with acid formation at more rapid rates, once the maximum period is initated. No P_H determinations were made within the initial two-hour interval, hence it is not possible to assume that any of the cultures showed an entire absence of the stationary period. In (4) and (5), however, the stationary period, if present, was probably of very short duration.

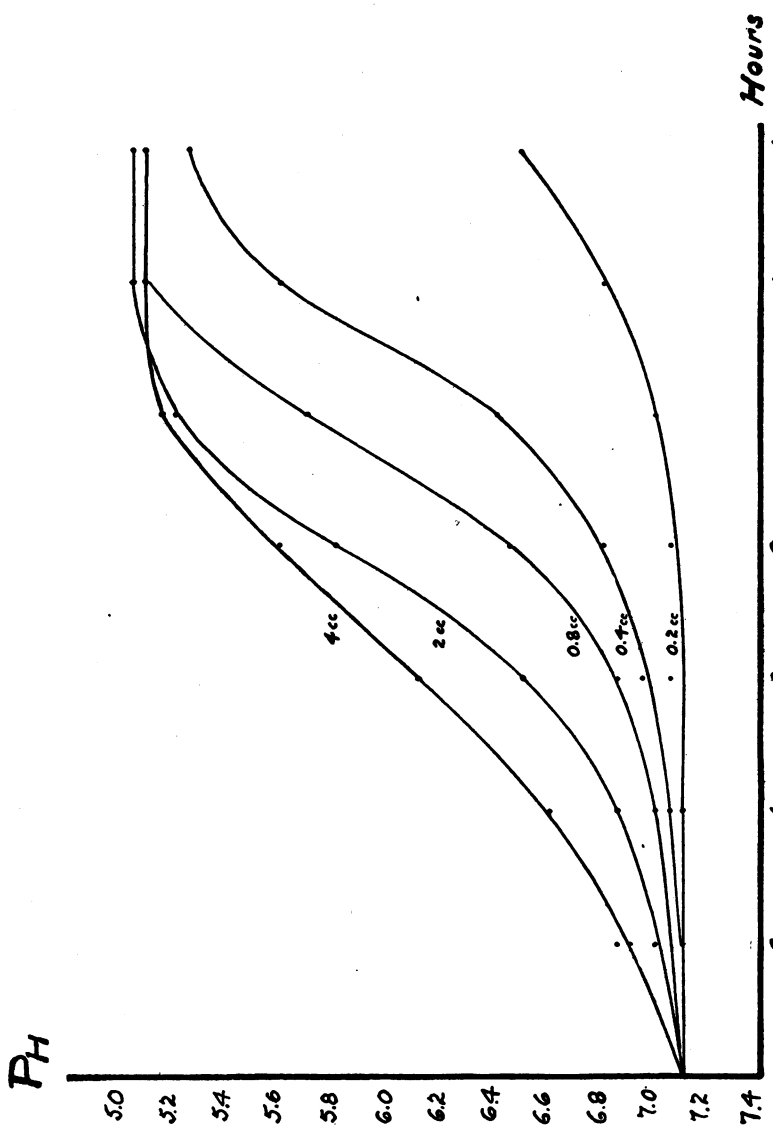

FIG. 3. EXPERIMENT VI. THE INFLUENCE OF THE AMOUNT OF INOCULUM UPON THE RATE OF ACIDITY FORMATION

Experiment VII. The relation of the age of parent culture to the rate of acid formation in glucose broth

Four cultures of *Streptococcus hemolyticus* were made in the usual manner at intervals of six hours. After eighteen hours incubation sub-cultures were made and these second-generation cultures incubated. The schedule was so arranged that at the time of the final inoculation into the medium of the experiment (20 cc. portions of 1 per cent glucose broth) organisms would be taken from parent cultures of six, twelve, eighteen and twenty-four-hours age. One hour previous to the final seeding counts of each parent culture were made by the method of Wright in order that each tube of broth to be used in the experiment might receive approximately the same number of organisms. The inoculum was based upon the proportion, 0.8 cc. of a twenty-four-hour culture per 20 cc. of broth.

Bacterial counts

NUMBER	AGE	ORGANISMS	INOCULUM
	hours	*millions per cu. mm.*	*cc.*
1	6	248	7.40
2	12	1088	1.68
3	18	1548	1.48
4	24	2282	0.80 (basis)

Examination of the curves (fig. 4) shows that (2) (from twelve hour culture) reaches the characteristic final P_H earliest, then come in order the tubes from the six-, eighteen- and twenty-four-hour parent cultures. The onset of the maximum period is seen to follow the same order. As might be expected, the differences are shown almost entirely in the duration of the lag and stationary periods of the four cultures. It is a fact of interest and importance that the rates of acid formation during the maximum period (table 6) were practically equal in the four cases.

From a consideration of the work of various investigators upon the life phases of an organism the results obtained here are not unexpected. It has been repeatedly demonstrated that the maximum rate of acid formation in glucose broth occurs between

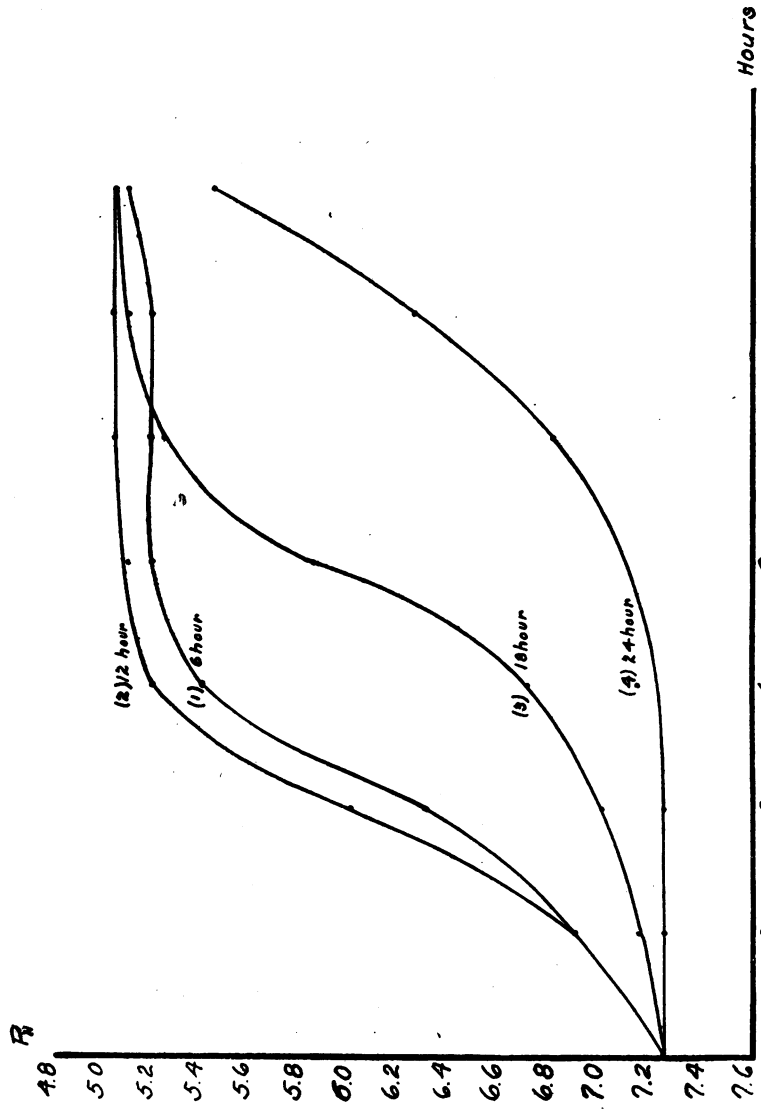

FIG. 4. EXPERIMENT VII. THE INFLUENCE OF THE AGE OF PARENT CULTURE UPON THE RATE OF ACIDITY FORMATION IN SUBCULTURES

the sixth and twelfth hours of incubation, provided the inoculum be taken from an eighteen-hour parent culture. If it be assumed that during this interval the organisms are growing rapidly and that their metabolic activities are at a maximum it would be anticipated that transplantation of organisms during this period to a favorable medium would result in resumption of growth and metabolism with a minimum of lag. The close parallelism in the curves of (1) and (2) bears out this supposition. That the organisms decrease progressively in vitality with the lengthening of their period of contact with the products of their own metabolism is brought out in the curves of (3) and (4).

TABLE 6

Experiment VII

NUMBER	AGE OF PARENT CULTURE	DURATION STATIONARY PERIOD	DURATION LAG PERIOD	ONSET OF MAXIMUM PERIOD	DURATION MAXIMUM PERIOD	P_H LOWERING (MAXIMUM PERIOD)	
						Total	Per hour
	hours	*hours*	*hours*		*hours*		
1	6	0	4	4	2	0.90	0.45
2	12	0	2	2	2	0.90	0.45
3	18	0	6	6	2	0.85	0.425
4	24	4	8	12	2 (?)	0.80	0.40

Here are seen more prolonged lag periods, indicating that the organisms required more time to recover from the injury sustained in the previous environment. The injury, however, appears to be only temporary for in all cases acid production is seen to proceed at practically the same rate following the onset of the maximum period. The entire absence of lag in acid production has never been observed with the streptococcus.

Experiment VIII. The rates of acid formation of Streptococcus hemolyticus in glucose broth and in glucose-serum broth

Forty cubic centimeter portions of infusion broth (initial P_H 7.20) containing (1) 1 per cent glucose, and (2) 1 per cent glucose plus 5 per cent horse serum were inoculated with 1.6 cc. of an eighteen-hour glucose broth culture and incubated at 37°. Determinations of hydrogen-ion concentration were made at

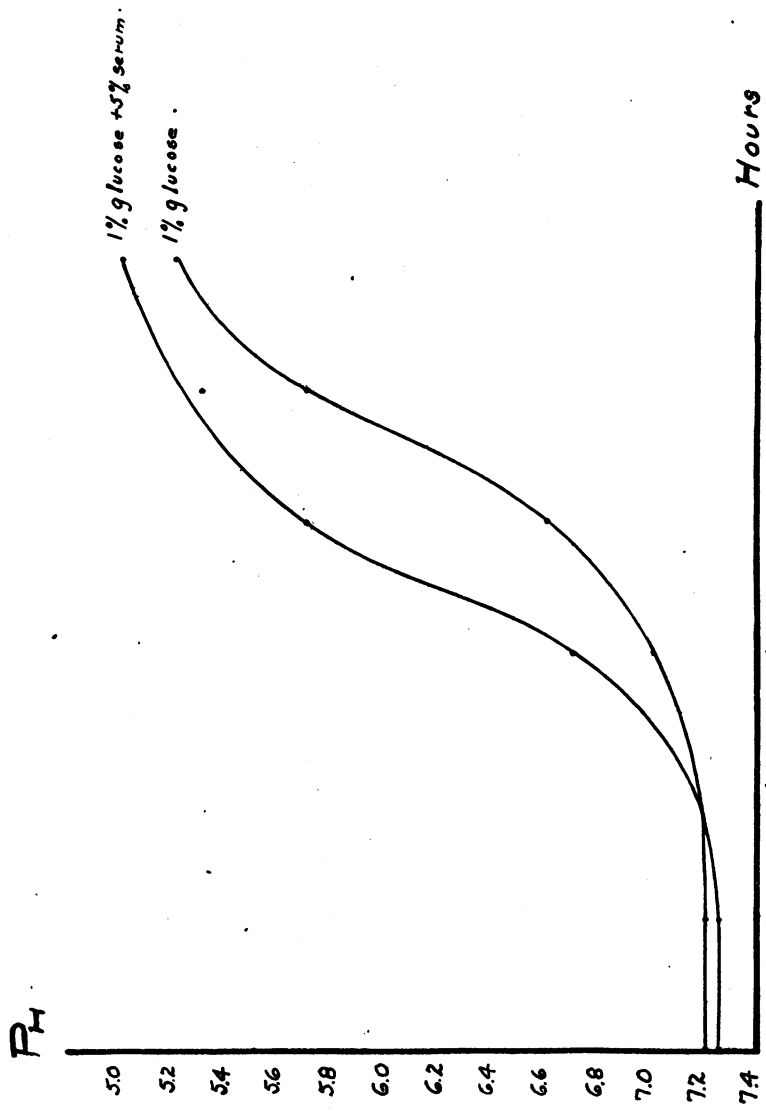

FIG. 5. EXPERIMENT VIII. THE RATE OF ACIDITY FORMATION IN GLUCOSE BROTH AND GLUCOSE-SERUM BROTH

the outset and at two-hour intervals by removing aseptically 2 cc. of material from the flasks. The experiment continued through twelve hours, at the conclusion of which period both cultures had reached their characteristic final level of P_H.

The outstanding fact here, as may readily be seen by reference to the curves (fig. 5), is a more rapid attainment of high levels of acidity on the part of the culture containing horse serum. Though a stationary period of two hours is noted in each, the lag in the glucose culture is of two hours longer duration than in the glucose-serum culture. A close parallelism in rates is seen during the maximum period.

It would seem logical to expect that the differences manifest in the above experiment would be closely correlated with the rates of increase in numbers of cells in the two cultures; in other words, multiplication at a maximum rate would be initiated earlier in the serum-glucose medium. It is a well recognized fact that we have at our disposal no very satisfactory method of enumerating viable streptococci. The method of Wright, though useful in the standardization of bacterial vaccines, gives only approximate results, and moreover, furnishes values which represent the total organisms, viable and nonviable, present in a culture. On the other hand, the method of plating dilutions of a culture which is recognized as valuable in numerical determinations of such organisms as *Bact. coli* and *Bact. typhosum*, is not adequate for enumerations of streptococci owing to the fact that single colonies upon the plate almost invariably represent streptococcal chains of varying length. Moreover, there arises a possibility of the breaking up of coccal chains through the mechanical disturbance occasioned in preparing dilutions of the culture.

Though the inadequacies of these two procedures were recognized it was nevertheless considered advisable to repeat experiment VIII supplementing the P_H determinations at two-hour intervals with estimations of the number of viable organisms through the medium of plate counts.

Experiment IX. The relationship between the rates of acid formation and growth of Streptococcus hemolyticus in glucose broth and in glucose-serum broth

Forty cubic centimeter portions of 1 per cent glucose broth and 1 per cent glucose-5 per cent horse-serum broth were prepared and incubated to insure sterility. Inoculations were made from an eighteen-hour, second-generation culture in 1 per cent glucose broth into the two lots of media. P_H determinations and plating of dilutions were carried out every two hours. The experiment continued through twelve hours.

Technic of plating. 1.8 cc. of plain broth were used as diluting fluid throughout. 0.2 cc. of culture was transferred into this amount of broth and the fluids mixed by carefully drawing up and down in the pipette, after which 0.2 cc. of this dilution were added to 1.8 cc. of broth, etc. until all the dilutions required had been made. Especial care was taken to avoid agitation of the material during the preparation of the dilutions. Nutrient agar containing 10 per cent of defibrinated rabbit's blood was used as a plating medium.

Table 7 contains the results of the experiment.

TABLE 7
Experiment IX

HOURS	GLUCOSE		GLUCOSE SERUM	
	P_H	Counts*	P_H	Counts*
0	7.65	1.68	7.65	1.68
2	7.65	0.38	7.65	40.30
4	7.60	158.00	7.20	140.00
6	7.50	1,498.00	5.90	76,000.00
8	6.80	3,243.00	5.15	713,600.00
10	5.90	250,000.00	5.05	Infinite
12	5.50	Infinite	4.90	1,040,000.00

* Counts are expressed in millions per cubic millimeter.

Attempts to construct growth curves by plotting the logarithms of counts against time brought out certain irregularities which made impossible the formation of smooth curves. Con-

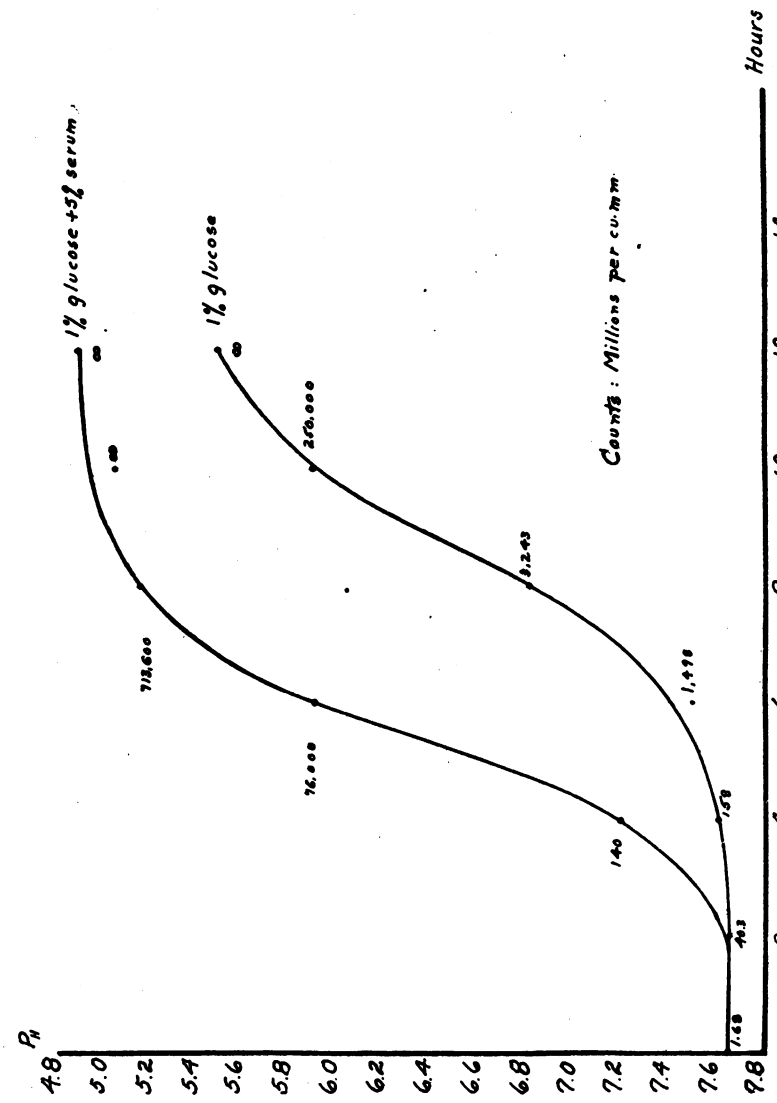

FIG. 6. EXPERIMENT IX. THE RATE OF GROWTH AND ACIDITY FORMATION IN GLUCOSE BROTH AND GLUCOSE-SERUM BROTH

sequently this procedure was abandoned. Curves of acid formation are shown in figure 6. At each point on the curves the number of organisms, expressed as millions per cubic millimeter, is shown. Examination of figure 6 shows that the two curves are analogous to those of experiment VIII (fig. 5), though the lag registered by the glucose-serum culture is of less duration. The numbers of viable organisms as shown by plate counts bear out the assumption that the earlier rise in acidity in a glucose-serum broth is associated with a corresponding period of multiplication at a rapid rate.

Experiment X. The relation of the initial P_H of glucose-broth to the rate of acid formation by Streptococcus hemolyticus

Beef infusion broth was adjusted to various P_H levels, divided into six portions, and sterilized in the usual manner. After adding the proper amount of glucose, the tubes, containing 20

TABLE 8

Experiment X

NUMBER	P_H (INITIAL)	DURATION OF STATIONARY PERIOD	DURATION OF LAG PERIOD	ONSET OF MAXIMUM PERIOD	DURATION OF MAXIMUM PERIOD	P_H LOWERING (MAXIMUM PERIOD)	
						Total	Per hour
1	5.20			No growth			
2	6.20	10		Not reached in 14 hours			
3	7.00	2	4	6th hour	2	0.85	0.425
4	7.50	2	2	4th hour	4	1.70	0.425
5	8.10	2	4	6th hour	2	1.25	0.625
6	8.65	2	?	Not reached	?		

cc. of medium each, were incubated to insure sterility. The inoculum consisted of 1.33 cc. of an eighteen-hour second-generation culture in 1 per cent glucose broth. A massive inoculum was employed to complete the experiment within the fourteen hours. The results are found in table 8.

Reference to figure 7 reveals an interesting point, namely, that the cultures of initial P_H 7.0, 7.5, 8.1 reached practically the same level of hydrogen-ion concentration after eight hours

incubation. To attain this result the cultures of necessity must have produced acid at varying rates. That this was true is brought out by the curves which show a tendency toward convergence after the second hour. From the data in table 8 it

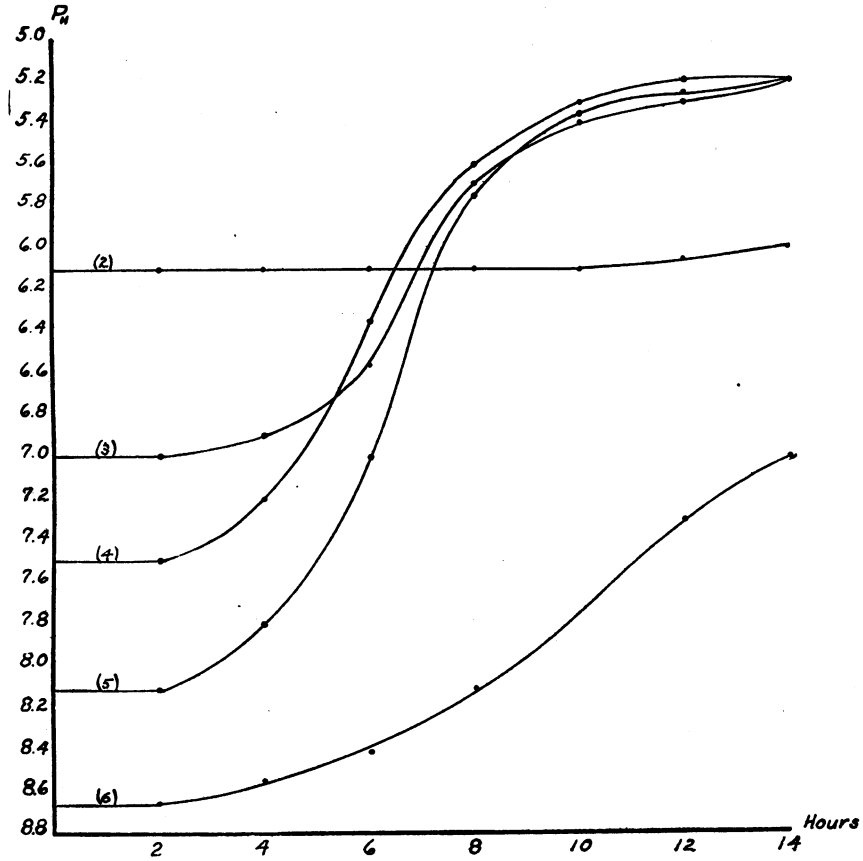

FIG. 7. EXPERIMENT X. THE INFLUENCE OF INITIAL P_H OF BROTH UPON THE RATE OF ACIDITY FORMATION

appears that culture (4) exhibited the shortest lag (two hours) though culture (5) showed the most rapid rate of acid formation during the maximum period, namely, a lowering of 0.625 P_H against a lowering of 0.425 P_H in the cases of (4) and (3). Culture

(6) began its acid formation after two hours at a slow, rather constant rate but at the close of the experiment had only reached a P_H of 7.0. After thirty hours its P_H was 6.0. It was not known whether this culture ever reached the final characteristic hydrogen-ion concentration. Culture (1) showed no growth while (2) was found to grow very poorly, the P_H after thirty hours being at the same level as at the fourteen-hour period.

TABLE 9

Summary

EXPERIMENT	AGE OF PLEURAL FLUID	DURATION STATIONARY PERIOD	DURATION LAG PERIOD	ONSET MAXIMUM PERIOD	P_H (INITIAL)	P_H CHANGE MAXIMUM PERIOD	DURATION MAXIMUM PERIOD	P_H LOWERING	
								Total	Per hour
Medium: 1 per cent glucose broth									
	days	*hours*	*hours*				*hours*		
VI	18	4	4	8	7.10	6.45–5.70	4	1.35	0.34
VII	19	Under2	6	6	7.25	6.70–5.85	2	0.85	0.425
VIII	18	3	5	8	7.20	6.60–5.70	2	0.90	0.45
IX	24	2	6	8	7.65	6.80–5.90	2	0.90	0.45
Medium: 1 per cent glucose, 5 per cent horse serum broth									
VIII	18	2	4	6	7.25	6.70–5.70	2	1.00	0.50
IX	24	2	2	4	7.65	7.20–5.90	2	1.30	0.65

From the foregoing data the following conclusions regarding the rate of acid formation in cultures of *Streptococcus hemolyticus* may be drawn:

1. The curves of acid formation with time may be separated into five characteristic periods: (1) Stationary period, (2) lag period, (3) maximum period, (4) period of negative acceleration, (5) maximum stationary period.

2. It is possible to reduce the duration of the stationary and lag periods to a minimum through increasing the quantity of inoculum. Whether this holds beyond a certain point is not known.

3. The age of the culture that is serving as a source of inoculum may exert a decided effect upon the duration of the stationary and lag periods in the sub-culture. If the inoculum be taken from a culture during its maximum period, lag is reduced to a

minimum in the sub-culture and growth and acid production at a maximum rate are initiated early. This point is of considerable importance, though seemingly it has been overlooked by many workers.

4. The presence of 5 per cent horse serum reduces lag by from two to four hours. This is correlated with an earlier period of multiplication of organisms at a maximum rate. Two possible explanations of this phenomenon present themselves: (1) Nutritive materials in some easily available form may be furnished by the serum or, (2) growth-accessory substances (vitamines) may be present in the enriching fluid. The second possibility would be in accord with Kligler's finding (1919) that the presence of tissue extracts shortened lag in the growth of *Streptococcus hemolyticus* and other organisms. Ordinarily these accessory substances are furnished by disintegrating cells which accounts for the fact that massive inocula give better cultures than light inocula.

5. Entire absence of lag in acid formation has never been noted. One case has been reported above in which a two-hour lag was apparent in glucose-serum broth.

6. In glucose broth the maximum period is initiated between the sixth and eighth hour and is usually maintained for two hours after which the period of negative acceleration sets in. The P_H decrease per hour in this medium is 0.42 (average of four experiments). In glucose-serum broth the maximum periods sets in two to four hours earlier and proceeds for two hours. The P_H decrease per hour during this period is 0.50.

Recent work in this laboratory by Dr. Marjorie W. Cook has demonstrated that hemotoxin production by the "H" strain of *Streptococcus hemolyticus* occurs nearly always between the sixth and eighth hours. It is a fact of interest that this property appears during the interval which is most frequently associated with maximum acid formation.

7. In glucose broth of initial P_H ranging from 7.10 to 7.65 the maximum period sets in when the P_H of the culture has been brought to 6.45–6.80. The relation of this level of acidity to the optimum P_H of the enzymes associated with acid production might be suggested as a possible explanation of this phenomenon.

8. The initial P_H of broth exerts an effect upon the rate of acid formation. A medium of P_H 7.5 was found to show a minimum of lag, while the most rapid acid formation occurred in broth of P_H 8.1. The optimum P_H of broth for growth and acid production of the "H" strain of *Streptococcus hemolyticus* apparently lies between these two points, P_H 7.5–8.1. Other observers have fixed the optimum P_H of the streptococcus at 7.8.

IV. THE INFLUENCE OF THE INITIAL P_H OF BROTH UPON GROWTH AND ACID FORMATION OF STREPTOCOCCUS HEMOLYTICUS

Before the elaboration of accurate methods for determining the true reaction of a medium much attention was given to the study of the influence of acidity and alkalinity upon the physiological activities of organisms. Unfortunately much of the data obtained in these earlier investigations is of little value owing to the fact that determinations of titratable acidity rather than of true acidity were carried out. The fallacy of titrating media by the older method has been established by Clark (1915a) beyond question and if we are to accept the classic works of Sörensen and Michaelis, as supplemented by a constantly increasing mass of data by other investigators, it must be supposed that the hydrogen-ion concentration rather than the titratable acidity of the environmental medium is the determining factor in regulating the metabolic activities of bacteria and related organisms.

Though it is true that media adjusted by the old titration method may vary considerably in their hydrogen-ion concentrations yet it has been possible in the past to cultivate bacteria with a considerable degree of success. No doubt this has been due rather to the fact that many bacteria are able to develop within a fairly wide range of reaction than to the accuracy of adjustment of the media. The effect of variations in initial P_H would be demonstrable rather in altered rates of growth and fermentation. In the case of some of the more delicate pathogenic bacteria, small variations in reaction may induce very decided effects and it is here particularly that the true reaction

must be carefully controlled. One example may serve to illustrate this point: H. M. Jones (1920) working with the various types of pneumococci found that in a medium of P_H 7.0 no strain was able to develop greater acidity than P_H 5.6, whereas if the initial reaction was P_H 7.6 all strains gave a final hydrogen-ion concentration ranging from 5.0 to 5.4. If the final P_H produced by certain organisms is to serve a useful purpose in differential procedures, the level of the initial hydrogen-ion concentration of the medium must obviously be controlled so as to permit the optimum development of the organism in question, in order that it may carry its fermentation to a maximum.

That there are levels of hydrogen-ion concentration which have the effect of limiting the activities of certain organisms was perhaps first recognized by Lazarus (1908) in 1908, who roughly adjusted her media to various hydrogen-ion concentrations with litmus, phenolphthalein, and methyl orange after which the reactions limiting growth were studied. The influence of reaction was considered a modification of the conditions of assimilation in that it exerted a definite effect upon the state of dissociation of the materials which the organism in question could take up or could alter.

With the recognition by investigators of the growing importance of the relationships of hydrogen-ion concentration to biological process in general, have come attempts to determine the limits of reaction within which bacteria may develop. The most complete single effort to establish such minimum, maximum, and optimum limits of P_H for a number of pathogenic organisms seems to have been that of Fennel and Fisher (1919). In the course of the present investigation it has been possible to collect from a number of sources data bearing on this point and in recognition of the value of a compilation such as this to workers in the field of bacteriology this information has been appended to the present section of the paper.

Experiment XI. The relation of initial hydrogen-ion concentration of broth to the growth of Streptococcus hemolyticus

Portions of infusion broth were adjusted to values ranging from P_H 5.0 to 9.0 and after the addition of proper amounts of glucose and horse serum, were incubated for twenty-four hours to insure sterility. Each tube contained 5 cc. of medium. The following series were used: (1) Plain broth, (2) 1 per cent glucose broth, (3) 1 per cent glucose-5 per cent horse serum broth. The inoculum consisted of 0.2 cc. of an eighteen-hour culture in 1 per cent glucose broth. Duplicate uninoculated tubes were carried as controls. The results are found in table 10.

The following summary will perhaps serve better to express the outstanding points of this experiment:

	PLAIN BROTH	1 PER CENT GLUCOSE-BROTH	1 PER CENT GLUCOSE, 5 PER CENT SERUM-BROTH
Minimum P_H permitting growth	6.35	6.35	5.70
Maximum P_H permitting growth	8.50+	8.50+	9.25+
P_H limits within which luxuriant growth occurs	6.60	6.35	5.90
	8.50	8.50	9.25

Whereas the limits of reaction which permit growth appear to be the same in plain and in 1 per cent glucose broth, the presence of horse serum in addition to the glucose enables the organisms to tolerate greater degrees of acidity and alkalinity. Hence it is to be emphasized that in expressing the levels of hydrogen-ion concentration which limit the growth of organisms the exact composition of the experimental media must be mentioned. It has been noted previously that horse serum exerts a strong stimulatory effect upon the growth and fermentative activities of the streptococcus. Here we find additional evidence of such an action in an increased tolerance of the organisms for acidity and alkalinity, manifested by growth throughout a wider range of hydrogen-ion concentration.

From the results of experiment VIII it must be concluded that the optimum P_H, based upon the rate of acid formation in 1

per cent glucose broth, lies between P_H 7.5 and 8.1. If the mean of these two exponents be taken, the value, P_H 7.8, represents the optimum hydrogen-ion concentration for growth and acid production. This corresponds to the optimum found by Fennel and Fisher (1919) for *Streptococcus hemolyticus*. It is interesting to note that this point corresponds exactly to the optimum established for the pneumococcus (see chart) and other pathogenic cocci, and that it is only slightly different from the P_H of human blood.

TABLE 10

Experiment XI

NUMBER	PLAIN BROTH P_H			1 PER CENT GLUCOSE BROTH P_H			1 PER CENT GLUCOSE, 5 PER CENT HORSE SERUM BROTH P_H		
	Initial	48 hours	Control	Initial	48 hours	Control	Initial	48 hours	Control
1	5.00	−	5.0	5.0	−	5.0	5.0		5.0
2	5.30	−	5.4	5.3	−	5.4	5.5	5.4±	5.7
3	5.50	−	5.55	5.5	−	5.55	5.7	5.1+	5.7
4	5.70	−	5.60	5.7	−	5.6	5.9	5.05+++	5.9
5	6.05	−	6.00	6.05	5.95±	6.0	6.3	5.00+++	6.3
6	6.35	5.5++	6.35	6.35	5.20++	6.35	6.4	5.00+++	6.4
7	6.60	6.0+++	6.70	6.60	5.15+++	6.70	6.80	5.00+++	6.80
8	7.00	6.15+++	6.95	7.00	5.25+++	6.95	7.00	5.00+++	7.05
9	7.15	6.40+++	−	7.15	5.20+++	−	7.20	5.00+++	7.20
10	7.45	6.60+++	7.30	7.45	5.1+++	7.30	7.50	5.00+++	7.40
11	7.85	6.80+++	7.50	7.85	5.25+++	7.50	7.70	4.90+++	7.65
12	8.10	7.00+++	−	8.10	5.20+++	−	8.10	5.00+++	8.10
13	8.35	6.90+++	8.25	8.35	5.20+++	8.25	8.30	5.00+++	8.30
14	8.70	8.10++	8.50	8.70	5.20+++	8.50	8.7	5.10+++	8.50
15	9.40	−	8.95	9.40	−	8.95	9.25	5.20++	8.90

− No growth; ± growth doubtful; + fair growth; ++ good growth; +++ excellent growth.

Wolf and Harris (1917a) working with *Clostridium welchii* and *C. sporogenes* have found that the final hydrogen-ion concentration produced by these organisms in media adjusted to different levels is by no means a constant. By constructing curves to show what they term "reaction resultants" an orderly relationship between the point of initial and final P_H was noted. Moreover, in media adjusted within the acid range the character of the "reaction resultant" curve was dependent upon the type of

acid employed in fixing the initial reaction of the medium. Further doubt has been thrown upon the "physiological constant" theory by the work of Wyeth (1918) on *Bact. coli*. By constructing "reaction resultants" such as those suggested by Wolf and Harris (1917a) he was able to show a definite relationship between the initial and final P_H levels. The type of acid employed in adjusting the medium was also found to bear a definite relationship to the final P_H produced by the organisms. From the foregoing results these investigators concluded that no method of clinical differentiation based upon the production of a characteristic level of hydrogen-ion concentration may safely be applied, unless such factors as the initial P_H of the culture medium as well as its composition be very carefully controlled in every test.

Wolf and Harris (ibid.) have directed attention to the fact that fermentations characterized by a slowly decreasing production of acid in the period of depressed acceleration give rise to a final P_H which is a constant regardless of the initial reaction, provided the activities of the organism cease as soon as a definite level of P_H is attained. Expressed differently, the "reaction resultant" appears as a straight line parallel to the abscissa. Seemingly this condition prevails in streptococcus fermentations as table 10 reveals a marked constancy in the levels of final P_H produced in glucose and in glucose-serum media. So far as the initial reaction is concerned it must be concluded that this factor is without influence upon the production of a characteristic hydrogen-ion concentration but that levels of initial P_H which allow growth to occur satisfactorily will also conduce to the attainment of the P_H level established as a "physiological constant" of *Streptococcus hemolyticus*. That the composition of the medium may exert an effect upon the final P_H however, is illustrated in the values obtained with the glucose-serum series (table 10). Here there is a tendency toward the production of slightly higher points of hydrogen-ion concentration, that is, lower P_H levels.

Limits of hydrogen-ion concentration which permit growth of organisms

ORGANISM	REFERENCE	MEDIUM	MINIMUM	MAXIMUM	OPTIMUM
Pneumococcus	Dernby and Avery (1918)	Infusion broth	7.0	8.3	7.8
	Fennel and Fisher (1919)	Infusion broth	7.2	8.2	7.8
	Avery and Cullen (1919b)	Infusion broth	7.0	8.3	7.8
Streptococcus hemolyticus	Fennel and Fisher (1919)	Infusion broth	4.5	8.0	7.6–7.8
	Foster	Infusion broth (1 per cent glucose)	6.35	8.5+	7.8
		Infusion broth (1 per cent glucose, 5 per cent cent horse serum)	5.7	9.25+	
		Infusion broth	6.35	8.5+	
Streptococcus viridans	Grace and Highberger (1920a)	Ascites broth	6.40	8.00	6.8
	Fennel and Fisher (1919)		4.50	8.00	7.6–7.8
Streptococcus erysipelatis	Itano (1916b)				2.24×10^{-8}
Meningococcus	Fennel and Fisher (1919)	Glucose-agar	7.40	7.80	7.6
	Gates	Serum-glucose broth	6.10	7.80	7.4
Gonococcus	Cole and Lloyd (1917)	"Tryptamine B. E."	6.50	9.10	7.6
	Fennel and Fisher (1919)	Starch-agar (Vedder)	7.0	8.00	7.6
Bact. coli	Michaelis and Marcora (1912)	Lactose broth	5.0		
	Shohl and Janney (1917)	Urine	4.6–5.0	9.2–9.6	6.0–7.0
	Wyeth (1918)	Infusion broth	4.30 (HCl)		
	Wyeth (1918)	Infusion broth	4.52 (lactic)		
	Wyeth (1918)	Infusion broth	4.77 (acetic)		

Limits of hydrogen-ion concentration which permit growth of organisms—continued

ORGANISM	REFERENCE	MEDIUM	MINIMUM	MAXIMUM	OPTIMUM
Bact. typhosum	Fennel and Fisher (1919)	Nutrient agar	4.00	9.60	6.2–7.2
	Schoenholz and Meyer (1919)		5.00	8.60	6.8–7.0
Bact. paratyphosum (A)	Fennel and Fisher (1919)	Nutrient agar	4.00	9.60	6.2–7.2
Bact. paratyphosum (B)	Fennel and Fisher (1919)	Nutrient agar	4.00	9.60	6.2–7.2
Bact. dysenteriae (Flexner)	Fennel and Fisher (1919)	Nutrient agar	4.80	9.60	6.2–8.4
Bact. dysenteriae (Shiga)	Fennel and Fisher (1919)	Nutrient agar	4.80	9.60	6.2–8.4
C. welchii	Wolf and Harris (1917a)	Glucose-peptone (2 per cent) water	4.8		
C. metchnikoff	Wolf and Harris (1917a)	Glucose-peptone (2 per cent) water	4.94		
Hemophilus influenzae	Fennel and Fisher (1917a)	Chocolate medium			7.8–8.0
Corynebact. diphtheriae	Bunker (1916–17)		6.30	8.20	6.5–7.5
V. cholerae	Fennel and Fisher (1919)	Extract agar or broth	5.60	9.60	6.2–8.0
B. melitensis	Fennel and Fisher (1919)	Nutrient agar	6.30	8.40	6.6–8.2

Reaction of differential media

OBSERVER	MEDIUM	PH (MINIMUM)	PH (MAXIMUM)	PH (OPTIMUM)
Fennel and Fisher (1919)	Endo			7.8–8.0
Kligler (1918)				7.8–8.0
Fennel and Fisher (1919)	Brilliant green	6.40	7.20	6.8–7.0
Kligler (1918)				7.0–7.2
Meyer and Stickel (1918)				6.4–7.0
Fennel and Fisher (1919)	Russel's double sugar	7.0	7.8	7.4–7.6
Kligler (1918)				7.4

V. THE RELATION OF HYDROGEN-ION CONCENTRATION TO INHIBITION AND DEATH OF STREPTOCOCCUS HEMOLYTICUS

It has long been noted that the growth of a microörganism beyond a certain point exhibits symptoms of inhibition, manifest first in a decreasing growth rate, second by complete cessation of growth, third by a definite decrease in numbers, and finally by death, at which point the culture becomes entirely sterile. Inhibition, representing as it does an almost universal bacteriological phenomenon, ensues from the toxic action of the products of its own metabolism upon the organism itself. Through the continuous accumulation of these waste products in the encompassing medium and through the inability of the organism to escape their contact inhibition becomes more and more pronounced and eventually death supervenes. If the metabolic products are largely of acid nature these substances will exert a harmful effect and if in greater concentrations, a fatal influence. This fact has been well illustrated in the curves of acid formation previously discussed.

Recognizing this principle, Kitasato (1888) in 1888 added various acids to neutral media and then determined the minimum dose required to kill *Bact. typhosum* and *V. cholerae*, and the maximum dose which would still permit their growth. As the results of these experiments were expressed only in terms of percentage concentration they have for us now only historical interest.

Paul and Krönig (1896, 1897) in 1896 pointed out that the toxicity of metallic salts for anthrax spores and for cells of *Staphylococcus aureus* is dependent chiefly upon the effect of the cation but that the anions and undissociated molecules as well may exert a certain influence. Strong acids were found to act in accordance with their concentration of hydrogen ions and to depend to a small extent upon the specific action of the particular anion or undissociated molecules. Winslow and Lockridge (1906) in an extensive study of the toxic effects of certain acids upon colon and typhoid bacilli found that strong acids such as HCl and H_2SO_4 proved fatal in concentrations at which they were highly ionized, whereas weak acids such as acetic and benzoic, proved fatal at concentrations where they were but slightly ionized. In the latter the effect appeared to be due rather to the whole molecules than to the actual concentration of hydrogen-ions.

Paul, Birstein, and Reuss (1910a) attributed a considerable toxic influence to the acid anion present as well as to the undissociated molecules. The toxic action of hydrogen-ions upon the cell appeared to be catalyzed by anions. This was found to be especially true of the weak organic acids. This finding has been supported by Norton and Hsu (1916) who added that the undissociated molecules act as negative catalyzers of the action of the hydrogen-ions. Addition of a salt having the same anion as the acid in question was found to decrease the disinfecting power through depression of the hydrogen-ion concentration (common ion effect), though the retarding influence appeared to be greater than would be expected from the decreased hydrogen-ion concentration alone. Salts not appreciably affecting the ionization of the acid brought about an increase in disinfecting power. These conclusions are not in accord with other results reported by Paul, Birstein, and Reuss (1910b) These observers showed that salts which exercised no disinfecting power in themselves were capable of increasing the toxicity of inorganic acids having the same or different anions.

A direct relationship between the degree of ionization of acids and their toxicity for yeast cells was reported by Bial (1902)

who accordingly divided the acids used into three classes based upon their ionization constants and similarity in toxicity. Surprising differences in the toxicity of various acids for molds were found by J. F. Clark (1899) in 1899. The degree of dissociation seemingly stood in no relation to the toxicity and this observer was forced to the conclusion that the inhibitory property, for molds at least, resided largely in the undissociated molecules.

The approximate concentrations of a number of common inorganic and organic acids required to inhibit growth of *Streptococcus pyogenes* have been determined by Taylor (1917) in the course of studies on the disinfection of war wounds. Considerable variation in potency was apparent with the organic acids investigated though apparently no account was taken of their degrees of ionization.

Wolf and Harris (1917a) in their study of the effect of acids upon the fermentations of *Clostridium welchii* and *C. sporogenes* point out that the influence is two-fold; first, that exerted by the hydrogen-ions, and second, that due to the anions and undissociated molecules. Lactic acid was found to have about the same toxicity as hydrochloric, whereas acetic, succinic, and butyric inhibited growth at lower hydrogen-ion concentrations (higher P_H). Wyeth (1918) reported similar results with *Bact. coli.* He points out that if the actual mass of acid be considered hydrochloric was more inhibitory than lactic or acetic acids but that the lethal points of such organic acids, in terms of hydrogen-ion concentrations, were lower than that of hydrochloric. In equivalent quantities the highly ionized acids proved more effective in inhibiting growth.

Lord (1919), has obtained data which lead him to believe that acidity is the principal inhibitory factor in glucose broth cultures of pneumococcus, though H. M. Jones (1920) very recently has succeeded in demonstrating that in the presence of body fluid such as blood serum or ascitic fluid the tolerance of this organism for hydrogen ions is considerably increased. This same phenomenon had been noted previous to the appearance of Jones' article during the course of the present investigation upon *Streptococcus hemolyticus* and the facts have proved so

interesting that they will be presented in this section of the paper.

In numerous experiments it has been observed that a glucose broth culture of *Streptococcus hemolyticus*, after reaching a stationary level of hydrogen-ion concentration during the first twenty-four hours, remains viable for a period varying from two to five days. Subcultures made on each succeeding day during this period of death show stationary and lag periods of increasing duration. To gain some idea of the factors contributing to this inhibition the following experiments were carried out:

Experiment XII. The growth and acid production of Streptococcus hemolyticus in neutralized filtrates

A transplant of the usual quantity of an eighteen-hour culture was made into 1 per cent glucose broth, P_H 7.5, and the material incubated until sterile (five days). The culture was then filtered through a sterile Berkefeld candle, after which the filtrate was brought back to the original reaction with sterile NaOH and re-inoculated with a fresh, actively growing culture. This procedure was repeated until no further growth resulted upon inoculation. The results are found in table 11.

TABLE 11
Experiment XII

FILTRATE	FINAL P_H	GROWTH	BROUGHT TO P_H
1	5.10	+++	7.60
2	5.00	+++	7.50
3	5.30	+++	7.80
4		−	

From the data shown in table 11, it would appear that acidity is the chief factor causing inhibition and death of the streptococcus in glucose broth cultures. The inhibition which finally appears may be due to two factors; first, to an exhaustion of nutrient materials in the medium, and second to the accumulation of toxic products other than acid which check metabolism and growth.

Chesney (1916) in a rather extensive investigation of the latent period of bacteria noted variations in the toxicity of filtrates, taken at intervals following the maximum period from plain broth cultures. Inhibition appeared strongest at the time when the culture had attained the summit of its growth and became progressively less as the period of incubation increased. At the point where the culture became sterile a minimum of inhibition was shown. Filtrates taken early in the maximum period of growth showed no inhibitory property while those taken near the end of the same period proved to be somewhat toxic. According to Chesney the inhibitory substances represent waste products of the bacterial cells or unused portions of food molecules, and the alteration of the cells occasioned by their exposure to these toxic materials is concerned with that structure or function which is essential to metabolism and hence to growth. It must be emphasized that in Chesney's experiments plain broth cultures were studied and that consequently the factor of acidity was absent. In fact no determinations of hydrogen-ion concentration were carried out.

It is a well recognized fact that plain broth cultures of the streptococcus remain viable throughout much longer periods than do glucose-broth cultures of the organism. This would tend to substantiate the conclusion drawn from experiment XII that acidity is the chief single factor causing inhibition and death of the streptococcus. Natvig (1909) in an investigation of acid production by the streptococcus arrived at the same conclusion.

Refrigeration of streptococcus cultures is known to be one of the best means of maintaining the viability of the organisms and it has been observed in this laboratory that such a procedure is especially useful in preserving the pleuritic exudates employed as a source of culture material in the present investigation. It would be expected that the decrease in temperature occasioned in transferring a culture from the incubator to the ice chest would reduce the rates of metabolism and growth to a low level. As a consequence the toxic products of bacterial metabolism would increase in the medium at a much slower rate than if the culture were incubated. Obviously this condition would tend to preserve the viability of a culture for long periods.

Experiment XIII. The inhibitory action of acids upon a culture of Streptococcus hemolyticus

One per cent glucose broth was inoculated as usual with an eighteen-hour actively growing culture and permitted to incubate for eighteen hours. At the end of this interval a portion was filtered with sterile precautions through a Berkefeld candle and another portion was centrifugalized. P_H determinations were then made upon the supernatant and the filtrate. Portions of beef infusion broth containing 0.5 per cent KH_2PO_4 (to aid in maintaining the reaction) and 0.5 per cent glucose were next adjusted to the P_H levels of the cultures, using the acids indicated in table 12. The supernatant fluid, Berkefeld filtrate, and tubes containing the broth adjusted with acids were inoculated with equal amounts of an eighteen-hour culture in 1 per cent glucose broth. Tests of viability were carried out by streaking one loopful of material on the surface of blood-agar plates at hourly intervals. As will be seen by reference to table 12 some of the tubes contained 5 per cent horse serum.

In the cases of (2) and (4) the addition of 5 per cent horse serum caused a change in P_H toward the alkaline side and consequently the results in these tubes are not comparable with the others. The rather close agreement in toxicity between lactic (1) and acetic (2) acids at the same P_H is of interest. The mixture of the two acids in molecular proportions killed in twelve hours, but inasmuch as the P_H of this tube was 5.15 as against 5.25 in (1) and (3) the result cannot be considered as evidence of increased toxicity. By comparing (6) with (1), (3), and (5) the protective action of horse serum is strikingly illustrated. Tube (6) contained viable cells after fifty-four hours contact with an acidity of 5.20; in other words, the streptococci were able to tolerate the same degree of acidity for a period nearly four times longer when in contact with 5 per cent horse-serum. Close agreement between the toxicities of the supernatant and filtrate are apparent ((7) and (8)) though neither proved as toxic as lactic or acetic acids of the same hydrogen-ion concentration.

TABLE 12

Experiment XIII

VIABILITY AFTER HOURS	ACID								
	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
	Lactic, P_H 5.25	Lactic, 5 per cent horse serum, P_H 5.40	Acetic, P_H 5.25	Acetic, 5 per cent horse serum, P_H 5.40	Lactic, acetic, P_H 5.15	Lactic and acetic, 5 per cent horse serum, P_H 5.20	Supernatant, P_H 5.20	Filtrate, P_H 5.30	Lactic, P_H 4.95
6	+++	+++	+++	+++	++	+++	+++	+++	+
7	+++	+++	+++	+++	++	+++	+++	+++	±
8	+++	+++	+++	+++	60++	+++	+++	+++	−
9	+++	+++	+++	+++	10+	+++	+++	+++	−
10	++	+++	+++	+++	6+	+++	+++	+++	−
11	+	+++	+++	+++	1−	+++	+++	+++	−
12	+	+++	++	+++	−	+++	+++	+++	−
13	±	+++	35++	+++	−	+++	+++	+++	−
14	−	+++	21++	+++	−	+++	+++	+++	−
15	−	+++	6+	+++	−	+++	+++	+++	−
16	−	+++	−	+++	−	+++	+++	+++	−
17	−	++	−	+++	−	+++	++	++	−
19	−	60++	−	++	−	+++	14+	13+	−
21	−	10+	−	60++	−	++	5+	12+	−
32	−	5+	−	24+	−	35+	−	−	−
36	−	4+	−	4+	−	23+	−	−	−
54	−	−	−	2±	−	7+	−	−	−

Numbers represent colonies developing from one loopful of culture.

+++ Profuse growth; ++ good growth; + growth sparse (less than 50 colonies); ± growth very doubtful (one or two colonies); − no growth after twenty-four hours incubation.

SUMMARY

1. Streptococcus hemolyticus is able to ferment the common hexoses and disaccharides but not the polysaccharides. The final hydrogen-ion concentration produced in broth containing different sugars varies between the limits P_H 4.85–5.40. The lowest P_H is registered in broth containing glucose; the highest P_H in broth containing lactose. The characteristic final P_H is seldom reached in the first generation but is usually attained by

the second generation culture. Subsequent transplants do not show lower levels of P_H.

2. Plain broth cultures of *Streptococcus hemolyticus* show a decrease in P_H which is practically the same as that exhibited by cultures of the organism in sugar-free broth. This is believed to be due to a selective action upon that portion of the peptone molecule which Pick has shown reacts typically like a carbohydrate.

3. *Streptococcus hemolyticus* is able to produce its characteristic final P_H in neutral broth containing 0.2 per cent glucose. Concentrations of glucose up to 1 per cent have no further effect upon the level of the final P_H.

4. The final hydrogen-ion concentration of the streptococcus is not influenced by the presence of K_2HPO_4 in concentrations up to 1 per cent providing sufficient glucose is present.

5. Titration curves show that horse serum in broth exerts a slight but distinct buffer effect.

6. The curves of acid formation with time may be separated into five characteristic periods; (1) stationary period, (2) lag period, (3) maximum period, (4) period of negative acceleration, and (5) maximum stationary period.

7. Through an increase in the amount of inoculum or by employing a parent culture of suitable age as a source of inoculum it is possible to reduce the stationary and lag periods to a minimum.

8. The presence of 5 per cent horse serum in glucose broth reduces lag in acid formation by two to four hours. This may be due to, (1) the presence of growth-accessory substances, or (2) the presence of easily available nutritive materials.

9. In glucose broth the maximum period of acid formation is initiated usually between the sixth and the eighth hours and is maintained for two hours. Maximum production of hemotoxin has been found to occur between the sixth and the eighth hours.

10. The most rapid formation of acid takes place in broth adjusted to a P_H of 8.1, while a minimum of lag is shown in broth of P_H 7.6. The optimum P_H for acid formation is believed to lie between these two levels, or at 7.8.

11. The limits of hydrogen-ion concentration which support growth of *Streptococcus hemolyticus* are as follows:

P_H	PLAIN BROTH	1 PER CENT GLUCOSE BROTH	1 PER CENT GLUCOSE, 5 PER CENT SERUM BROTH
Minimum permitting growth	6.35	6.35	5.70
Maximum permitting growth	8.50+	8.50+	9.25+
Limits permitting luxuriant growth	6.60−	6.35−	5.90−
	8.50	8.50	9.25

12. Acidity is the chief factor contributing to inhibition and death of the streptococcus in glucose broth cultures. This is evidenced by the fact that growth proceeds luxuriantly in filtrates from active cultures the acidity of which has been neutralized by a base.

13. At a P_H of 5.25 lactic and acetic acids appear to have about equal disinfecting powers for *Streptococcus hemolyticus*. Organisms live for longer periods in filtrates from active cultures than in broth brought to the same P_H with either lactic or acetic acids.

14. A marked increase in tolerance for acid is shown by streptococci in the presence of horse serum. In one test it was found that viability persisted for a period nearly four times as long in serum-glucose broth of P_H 5.20 as was evident in glucose broth adjusted to the same P_H.