THE OPTIMUM HYDROGEN ION CONCENTRATION FOR THE GROWTH OF PNEUMOCOCCUS.

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(Received for publication, May 23, 1918.)

It is well known that pneumococcus grows best in slightly alkaline medium, but the exact degree of alkalinity, in terms of hydrogen ion concentration, necessary for optimum growth does not appear to have been determined. The work of Clark (1915) and Clark and Lubs (1917) makes it probable that the limiting and optimum hydrogen ion concentrations for the growth of bacterial races are as definite and characteristic as those for enzyme action. It consequently appears desirable to fix these values for such bacteria as we attempt to cultivate under accurately reproductible conditions. In the work here presented the attempt has been made to ascertain the optimum and limiting hydrogen ion concentrations for the growth of different types of pneumococcus.

Methods.

The substrate was plain salt-free broth, made up as follows: 1 pound of lean chopped beef is allowed to infuse in a liter of tap water over night on ice. The unfiltered meat infusion is boiled for 30 minutes, filtered through paper, and the loss by evaporation made up by the addition of water. 1 per cent peptone is added. The mixture is allowed to boil for 2 minutes. Sufficient sodium hydroxide is then added to turn the solution slightly alkaline to phenolphthalein. The medium is boiled for 6 or 7 minutes, made up to volume, filtered clear, and sterilized in an Arnold sterilizer for 20 minutes on 3 successive days. During this process it becomes less alkaline so that 0.3 to 0.5 cc. of normal sodium hydroxide is required to make 100 cc. neutral to phenolphthalein. The reaction is slightly alkaline, the value for pH lying between 7.6 and 7.9.

Bacterial growth was first estimated by plating. Later it was found that comparative bacterial counts could be made accurately and much more easily by estimating the turbidity of the broth suspensions with a Kober nephelometer. The nephelometric method has already been used by Dreyer and Gardner for this purpose. Its use is limited to solutions with reactions between pH = 6.0 and 8.0, because below a pH of 5.0 some proteins or peptones are precipitated, and above a pH of 8.0 phosphates may precipitate.

We attempted to standardize the method by means of silver chloride suspensions, but they did not prove to be satisfactory standards. The standard solution used was a suspension of dead pneumococci in which the number of cocci per cubic centimeter had been estimated by the plate method before the culture was killed. Because of the difference in color between standard suspension and broth culture, as well as the uncertainty in the absolute count of the standard, the results make no claim to absolute accuracy in the number of bacteria per cubic centimeter. Good comparative results were obtained, however, by comparing all the cultures of a given series with the same standard suspension. The latter was diluted in each instance so that a column of 20 mm. corresponded in turbidity to a column of 15 to 30 mm. of the broth culture.

With a given standard the depth at which the column of a culture must be set in the nephelometer does not vary accurately in inverse ratio to the number of cocci per cubic centimeter of culture. The formula suggested by Kober, however, could not be applied. The relation is expressed by a curve rather than a straight line. This curve was plotted after making a number of control observations in which the standard suspension was compared with known dilutions of itself, and the curve obtained was used as a basis for graphic calculation of results. This is the method that Bloor used in nephelometric determinations of phosphorus.

Hydrogen ion concentrations of the cultures were determined by Sörensen's colorimetric method with some of the modifications suggested by Clark and Lubs. Sörensen's phosphate solutions used as buffers were made by diluting stock 0.5 M solutions of KH_2PO_4 and $Na_2HPO_4.2H_2O$ (Kahlbaum's preparations made according to Sören-

sen). The indicators used were those recommended by Clark and Lubs.[1]

Standard solutions used:

$\frac{M}{15}$ disodium phosphate, $Na_2HPO_4 . 2H_2O$.[2]

$\frac{M}{15}$ monopotassium phosphate, KH_2PO_4.

Indicators used:

Name.	Range of pH.
Dibromo-*o*-cresolsulfonephthalein ("bromocresol purple")	5.2–6.8
Dibromothymolsulfonephthalein ("bromothymol blue")	6.0–7.6
Phenolsulfonephthalein ("phenol red")	6.8–8.4
o-Cresolsulfonephthalein ("cresol red")	7.2–8.8
o-Cresolphthalein	8.2–9.8

For the pH determinations the broth cultures were diluted with two volumes of carbon dioxide-free distilled water, and compared with the standards in the rack shown in Text-fig. 1 which is similar to that used by Walpole and then adopted by Clark and Lubs, but apparently somewhat more convenient. The high color of medium, which in itself might interfere with accurate readings, was compensated by using the comparator method in which the color of a broth control is superimposed upon that of the indicator. The values of pH could be easily determined within a limit of error of 0.05.

All experiments were carried out as follows: Triplicate 5 cc. portions of salt-free broth were placed in test-tubes with 1 cc. portions of buffer solutions to fix the reaction. The tubes were then resterilized. Of each set of three, two were inoculated with 0.1 cc. each of an 18 hour broth culture of pneumococcus while the third served as a control.

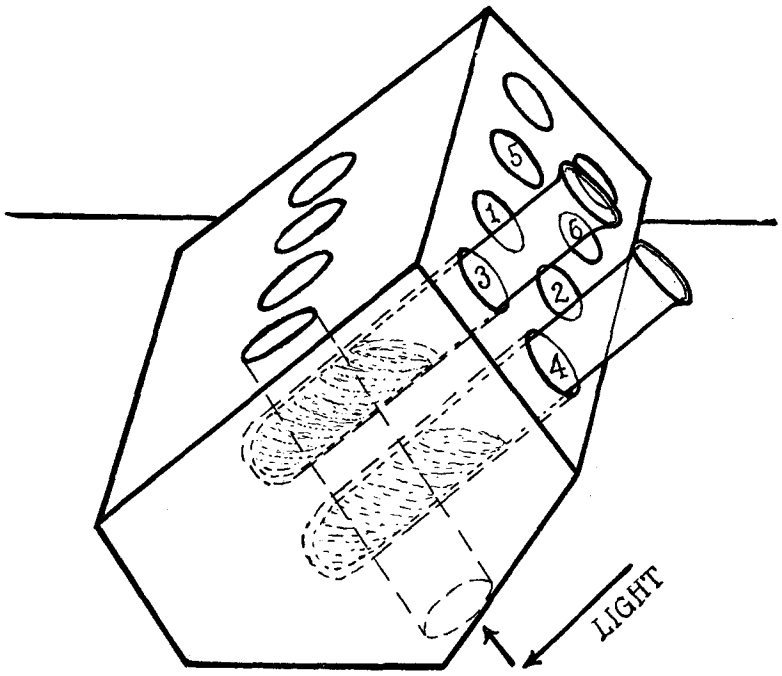
The tubes were incubated at 37°C. for 4 to 48 hours. Portions were taken for bacterial count when the latter was made by plating. The remainder of each culture was then sterilized by heat and the hydrogen ion concentration determined. When the bacterial count was estimated by nephelometer, the entire culture was sterilized before estimating either count or pH.

[1] The indicators were prepared by Hynson, Westcott, and Dunning, Baltimore.

[2] The purity of the salts was verified with the hydrogen electrode by Dr. Glenn E. Cullen.

It may be questioned whether the sterilization by heat in itself affects the hydrogen ion concentration of the broth medium. In order to determine this point the following experiment was devised.

To 5 cc. of uninoculated medium was added 1 cc. of buffer solution as described above. Each solution was then divided into two portions



TEXT-FIG. 1. Walpole's comparator modified to use reflected light. Tube 1, culture; No. 2, water; Nos. 3 and 5, indicator solutions of known hydrogen ion concentration; Nos. 4 and 6, diluted broth controls without indicator.

of 3 cc. each. One portion was sterilized for 20 minutes at 15 pounds pressure in an autoclave and then cooled to room temperature. The other portion served as control. The following results were obtained.

	pH				
After sterilization	7.0	7.4	7.6	8.0	8.8
Control	7.0	7.4	7.6	8.0	8.6

From this experiment it is evident that within the range of pH which we have used in our experiments, no essential change in pH was brought about by sterilization. At greater alkalinities, however, a slight variation seems to occur.

EXPERIMENTAL.

As electrolyte buffers were to be used to fix the hydrogen ion concentrations, it was a necessary preliminary to determine the effect of salt concentration by itself on the growth of pneumococci, in order that buffer concentrations might with certainty be avoided which would retard growth by their mere salt effect or osmotic pressure. Consequently the experiment recorded in Table I was performed.

TABLE I.

Influence of the Salt Concentration on the Growth of Pneumococci (Type I). Sodium and Potassium Chlorides.

Initial pH = 7.9
Incubation: 18 hours at 37°C.

Tube No.	Molar concentration of salt.	Growth.	
		NaCl	KCl
	mols		
1	1.0	−	−
2	0.4	−	−
3	0.2	−+	−+
4	0.1	++	++
5	0.04	++	++
6	0.01	++	++
7	Salt-free broth.	++	++

Concentrations over 0.1 M retard growth, and 0.4 M inhibits it altogether. There seems to be no essential difference in effect between sodium and potassium ions. For this reason we appear justified in using the salts $Na_2HPO_4.2H_2O$ and KH_2PO_4 as reaction regulators without regard to the interchange of potassium and sodium in different solutions.

The results in Table I demonstrate that the osmotic pressure of 0.1 M sodium chloride solution does not retard growth. Since Na_2HPO_4

dissociates into three ions, as compared with the two ions of NaCl, the phosphate solution of corresponding osmotic concentration is approximately $\frac{2}{3} \times 0.1$ M = 0.067 M. In order to stay safely within this limit and rule out osmotic concentration as a factor, the phosphate ion concentration in the cultures was kept down to 0.05 M. The results in Table II indicate that this phosphate concentration is well below that which noticeably retards growth.

TABLE II.

Influence of the Phosphate Concentration on the Growth of Pneumococci (Type I).

Phosphate mixture: 9 parts of Na_4HPO_2 + 1 part of KH_2PO_4.
pH = 7.7
Incubation: 18 hours at 37°C.

Tube No.	Molar concentration of phosphates.	Growth.
	mols	
1	0.8	−
2	0.4	±
3	0.2	+
4	0.08	++
5	Salt-free broth.	++

TABLE III.

Hydrogen Ion Concentration of Mixtures of Plain Broth, Phosphates, and Hydrochloric Acid or Sodium Hydroxide.

10 cc. of salt-free broth + 1 cc. 0.5 M phosphates + HCl or NaOH + water = 12 cc.

Tube No.	Added.				Hydrogen ion concentration of the mixture.
	0.1 N HCl	0.1 N NaOH	0.5 M KH_2PO_4	0.5 M Na_2HPO_4	
	cc.	*cc.*	*cc.*	*cc.*	*pH*
1	1.2	−	0.5	0.5	6.8
2	0.8	−	0.7	0.3	7.1
3	0.4	−	0.8	0.2	7.3
4	−	−	0.85	0.15	7.5
5	−	−	0.9	0.1	7.7
6	−	0.2	0.93	0.07	7.9
7	−	0.6	0.97	0.03	8.2
8	−	1.2	1.0	0.0	8.6

Table II shows that phosphates by their mere salt effect retard growth in about the same concentration as chlorides.

In Table III the results are recorded of a preliminary experiment performed in order to ascertain the amount of acid, alkali, or phosphate necessary to add to the broth in order to obtain hydrogen ion concentrations over the desired range.

It is known that the pneumococcus in glucose-containing solution is an acid producer. The results in Table IV show the effect of the acid formed on the hydrogen ion concentration of an ordinary broth. The final pH of 6.9 attained after 48 hours is, as shown by later experiments, in itself sufficient to stop growth.

TABLE IV.

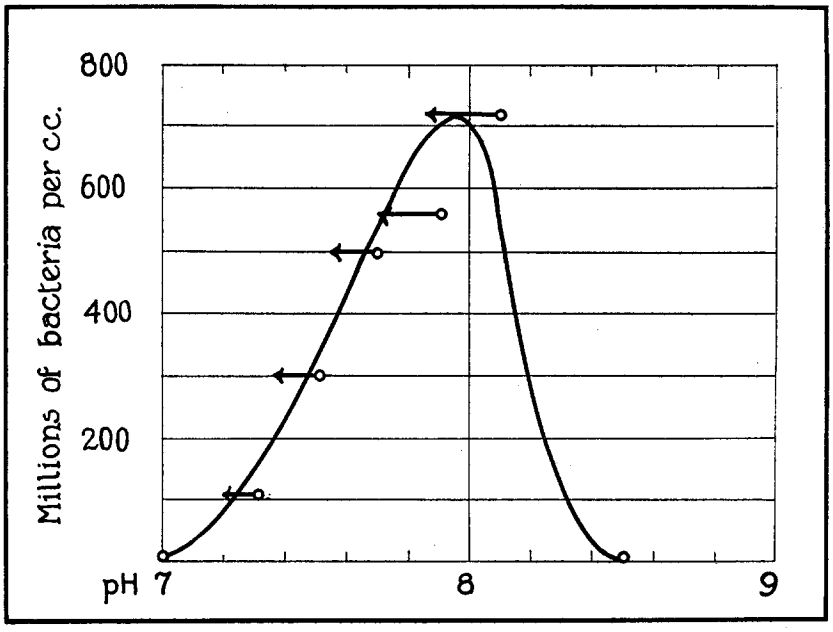
Change in the Hydrogen Ion Concentration of Plain Broth without Buffer Due to the Growth of the Pneumococci.

Tube No.	Length of incubation. (37°C.)	Hydrogen ion concentration.	Growth.
	hrs.	*pH*	
1	3	7.8	–
2	4	7.4	+
3	8	7.35	++
4	24	7.2	++
5	48	6.9	++

The results of Table IV show that buffers must be used to prevent changes in pH so great that results cannot be accurately interpreted, while Table II shows that the concentration of the phosphate buffers must not exceed 0.1 M. With further experience it was found desirable to keep the total phosphate concentration down to 0.05 M.

Results obtained in growing different types of pneumococci in solutions of definite pH, as fixed by phosphate buffers and controlled by colorimetric observations, are given in Tables V to X. The results are summarized graphically in Text-fig. 2.

It is evident from the above results that the optimum hydrogen ion concentration for the growth of pneumococci lies at a pH of about 7.8. It seems to be the same for various types of pneumococcus.



TEXT-FIG. 2. Relation between pneumococcus growth and pH of medium. The arrows indicate the shift in pH towards the acid side which occurred during growth, despite the presence of phosphate buffers. This change is much greater in ordinary media, where phosphates are not added.

TABLE V.

Determination of Optimum Hydrogen Ion Concentration. Pneumococcus Type I.

5 cc. of salt-free broth + 0.5 cc. of 0.5 M phosphates + NaOH or HCl + 0.1 cc. of bacterial culture + water = 6 cc.

Type I after 11 hours at 37°C.

Incubation: 4 hours at 37°C.

Growth determined by the plate method.

Tube No.	Hydrogen ion concentration.		Growth.
	Initial.	After 4 hrs.	
	pH	*pH*	
1	7.3	7.3	6,000,000 per cc.
2	7.6	7.35	5,000,000 " "
3	7.85	7.65	38,000,000 " "
4	8.1	7.9	25,000,000 " "
5	8.3	8.0	2,000,000 " "
6	8.6	8.4	1,000,000 " "

TABLE VI.

Determination of Optimum Hydrogen Ion Concentration. Pneumococcus Type I.

5 cc. of salt-free broth + 0.5 cc. of 0.5 M phosphates + NaOH or HCl + 0.1 cc. of bacterial culture + water = 6 cc.

Type I after 18 hours at 37°C.

Incubation: 20 hours at 37°C.

Growth determined by the plate method.

Tube No.	Hydrogen ion concentration.		Growth.
	Initial.	After 20 hrs.	
	pH	*pH*	
1	6.8	6.8	1,800 per cc.
2	7.0	7.0	1,700 " "
3	7.25	7.15	900,000,000 " "
4	7.45	7.3	400,000,000 " "
5	7.65	7.4	1,700,000,000 " "
6	7.9	7.65	1,400,000,000 " "
7	8.2	7.75	550,000,000 " "

TABLE VII.

Determination of Optimum Hydrogen Ion Concentration. Pneumococcus Type I.

5 cc. of salt-free broth + 0.5 cc. of 0.5 M phosphates + NaOH or HCl + 0.1 cc. of bacterial culture + water = 6 cc.

Type I.

Incubation: 5 hours at 37°C.

Growth nephelometrically determined.

Tube No.	Hydrogen ion concentration.		Growth, determined by the nephelometric method.
	Initial.	After 5 hrs.	
	pH	*pH*	
1	6.9	6.9	0
2	7.15	7.15	27,000,000 per cc.
3	7.3	7.3	52,000,000 " "
4	7.5	7.5	73,000,000 " "
5	7.7	7.6	79,000,000 " "
6	7.9	7.8	120,000,000 " "
7	8.3	8.2	0 " "
Control without phosphates....	7.8	7.4	100,000,000 " "

TABLE VIII.

Determination of Optimum Hydrogen Ion Concentration. Pneumococcus Type II.

5 cc. of salt-free broth + 0.5 cc. of 0.5 M phosphates + HCl or NaOH + 0.1 cc. of bacterial culture + water = 6 cc.

Type II.

Incubation: 18 hours at 37°C.

Growth nephelometrically determined.

Tube No.	Hydrogen ion concentration.		Growth, determined by the nephelometric method.
	Initial	After 18 hrs.	
	pH	*pH*	
1	6.8	6.8	0
2	7.0	7.0	0
3	7.3	7.15	450,000,000 per cc.
4	7.5	7.3	500,000,000 " "
5	7.7	7.5	800,000,000 " "
6	7.9	7.6	900,000,000 " "
7	8.3	8.3	Tr.
8	8.6	8.5	0
Control without phosphates....	7.8	7.1	700,000,000 " "

TABLE IX.

Determination of Optimum Hydrogen Ion Concentration. Pneumococcus Types I, II, and III.

5 cc. of salt-free broth + 0.5 cc. of 0.5 M phosphates + HCl or NaOH + 0.1 cc. of bacterial culture + water = 6 cc.

Incubation: 22 hours at 37°C.

Growth nephelometrically determined.

Tube No.	Initial hydrogen ion concentration.	Type I.		Type II.		Type III.	
		Hydrogen ion concentration after 22 hrs.	Growth per cc.	Hydrogen ion concentration after 22 hrs.	Growth per cc.	Hydrogen ion concentration after 22 hrs.	Growth per cc.
	pH	*pH*		*pH*		*pH*	
1	7.0	7.0	0	7.0	0	7.0	Tr.
2	7.3	7.2	110,000,000	7.15	280,000,000	7.15	310,000,000
3	7.5	7.35	300,000,000	7.3	440,000,000	7.3	440,000,000
4	7.7	7.45	520,000,000	7.4	500,000,000	7.4	520,000,000
5	7.9	7.6	560,000,000	7.6	850,000,000	7.6	680,000,000
6	8.1	7.85	720,000,000	7.85	800,000,000	7.85	500,000,000
7	8.5	8.5	0	8.5	0	8.5	0

TABLE X.

Determination of Optimum Hydrogen Ion Concentration. Pneumococcus Type II.

5 cc. of salt-free broth + 0.5 cc. of 0.5 M phosphates + NaOH or HCl + 0.1 cc. of bacterial culture + water = 6 cc.

Incubation: 18 hours at 37°C.

Growth determined nephelometrically.

Tube No.	Initial hydrogen ion concentration.	Type IIa (Jones).		Type II.		Type IIc.	
		Hydrogen ion concentration after 18 hrs.	Growth per cc.	Hydrogen ion concentration after 18 hrs.	Growth per cc.	Hydrogen ion concentration after 18 hrs.	Growth per cc.
	pH	*pH*		*pH*		*pH*	
1	6.8	6.8	0	6.8	0	6.8	0
2	7.1	7.1	120,000,000	7.1	0	7.1	0
3	7.2	7.2	140,000,000	7.2	140,000,000	7.25	Tr.
4	7.5	7.4	160,000,000	7.4	160,000,000	7.4	150,000,000
5	7.65	7.5	160,000,000	7.5	170,000,000	7.5	160,000,000
6	7.85	7.65	200,000,000	7.7	200,000,000	7.65	220,000,000
7	8.15	8.0	180,000,000	8.0	200,000,000	8.0	170,000,000
8	8.5	8.5	0	8.5	0	8.5	0
Control without phosphates.....	7.8	7.3	200,000,000	7.2	130,000,000	7.2	200,000,000

The optimum reaction is thus slightly more alkaline than the normal hydrogen ion concentration of blood. Growth does not occur in media in which the reaction is more acid than indicated by a pH of 7.0, which is practically the neutral point, or in media of alkalinity greater than indicated by a pH of 8.3. The range of hydrogen ion concentration within which pneumococci grow is consequently a narrow one.

The practical conclusion from these results is that the reaction of culture media for pneumococci should be fixed at a pH of 7.8 to 8.0. Broth with a pH less than 7.6 should not be used. In regulating the

reaction, acid or alkali should be added until direct pH determinations show that the proper reaction has been obtained.[3]

If phosphate solutions are used to assist in regulating the reaction, their concentration in the medium should not exceed 0.1 M, and no other salt should be added.

SUMMARY.

1. The optimum hydrogen ion concentration for the growth of the various types of pneumococcus is a pH of about 7.8.

2. The limiting hydrogen ion concentrations for the growth of pneumococcus are a pH of 7.0 and a pH of 8.3.

3. Phosphates used in adjusting reactions of media retard growth if present in a concentration greater than 0.1 molecular.

4. Culture media for pneumococci should, therefore, have an initial reaction between a pH of 7.8 and 8.0 and a total salt concentration not exceeding 0.1 M.

[3] At present, bouillion for growth of pneumococcus is prepared in this laboratory as described under "Methods," with the exceptions that 0.5 per cent of NaCl is added, and sufficient sodium hydroxide to bring the reaction to a pH of 7.8 to 8.0

The standard solutions required for the range within which media for growth of pneumococcus should fall are the following:

		pH
1.	8.8 cc. of $\frac{M}{15}$ Na_2HPO_4 + 1.2 cc. of $\frac{M}{15}$ KH_2PO_4	7.6
2.	9.2 " " $\frac{M}{15}$ Na_2HPO_4 + 0.8 " " $\frac{M}{15}$ KH_2PO_4	7.8
3.	9.5 " " $\frac{M}{15}$ Na_2HPO_4 + 0.5 " " $\frac{M}{15}$ KH_2PO_4	8.0
4.	9.7 " " $\frac{M}{15}$ Na_2HPO_4 + 0.3 " " $\frac{M}{15}$ KH_2PO_4	8.2

Phenol red or cresol red in 0.2 per cent solutions are satisfactory indicators. If the broth samples used for the pH determination are strongly colored, they may be diluted with two volumes of distilled carbon dioxide-free water, without producing any essential change of the hydrogen ion concentration.

This method of standardizing media has already proved itself valuable. It has practically eliminated the frequent irregularity of growth of pneumococcus media the reaction of which formerly was determined only by titration.

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