THE EFFECT OF HYDROXYL ION CONCENTRATION ON THE THERMAL DEATH RATE OF BACTERIUM COLI[1]

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INTRODUCTION

Strong acids and bases are known to be germicidal to bacteria. The growth limiting concentrations of hydrogen and hydroxyl ions have been determined for the more common organisms. Also, a number of investigations have been made on the effect of hydrogen ion concentration in accelerating the death rate of bacteria exposed to heat. This problem has been of particular interest to the canning industry. Less heat, it has been found, is necessary for complete sterilization of fruits and vegetables which are highly acid than for those which are more nearly neutral in reaction.

Less is known about the effect of alkalinity on the death rate of bacteria exposed to heat. Interest has arisen in this particular problem because of its bearing on the washing and sterilization of milk bottles and utensils. It would be of value in this connection to know just how important a factor the degree of alkalinity of the washing solution is.

It has been the purpose of this study to determine the magnitude of the effect of alkalinity in the destruction of bacteria. As a means of measuring the germicidal action of the hydroxyl ion,

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the effect of the pH of the medium of exposure on the thermal death rate of *Bacterium coli* has been studied.

HISTORICAL

As pointed out by Clark (1925), the results of many investigations have shown that the disinfectant action of an alkali is, in large measure, due to the hydroxyl ion concentration. A distinction must be made, however, between the more direct action of the hydroxyl ion concentration on bacterial cells, as in the case of strong bases, and the control of the effective state of a toxic compound by the concentration of hydroxyl ions.

Cohen (1922) showed how the viability of *Bacterium typhosum* in dilute buffer solutions was affected by various hydrogen ion concentrations. Cultures of *Bacterium typhosum* were planted in M/500 buffer solutions adjusted to pH values from 3.8 to 9.5 and these were incubated at 20°C. At all hydrogen ion concentrations decline in numbers occurred, but the least occurred at pH 5.0 and pH 5.4. At pH 3.8 the death rate was the greatest, the culture dying in six hours. At pH 9.5 the death rate was also rapid, the culture dying after twenty-four hours. The death rate, this experiment shows, is increased by a high pH value as well as by a low one; or, the death rate increases with an increase in hydrogen ion concentration below pH 5.0 and increases with an increase in hydroxyl ion concentration above pH 5.4.

The effect of the pH of the medium on the time necessary to destroy spores exposed to a high temperature has been studied by Bigelow and Estey (1920). They found that the thermal death rate was accelerated by an increased hydrogen ion concentration. At a temperature of 120°C. the time necessary to destroy spores of some thermophilic organisms in a medium of pH 4.6 was about one-fourth the time necessary for destruction in a medium of pH 6.0. The hydrogen ion concentration in this case caused a marked acceleration in the thermal death rate.

Chick and Martin (1912) have shown that an increase in the concentration of hydroxyl ions increases the denaturation rate of egg albumen in alkaline solutions. Although the coagulation of proteins of the type studied is probably different in many ways

from the destruction of bacterial cells, this work is no doubt a great help in understanding the process which takes place when the bacterial cell is destroyed by heat. It was found that, as the reaction proceeds, hydroxyl ions are continuously removed from solution.

Weiss (1921) showed that the thermal resistance of spores of *Clostridium botulinum* was lowered as the hydroxyl ion concentration was increased. At any given temperature, in a range between 95°C. and 120°C., the time necessary to destroy the spores at a pH of 8.2 was several times that necessary for destruction at a pH of 11.4.

From these investigations we would expect that the thermal death rate of *Bacterium coli* would be accelerated by an increased concentration of hydroxyl ions.

EXPERIMENTAL

Buffer mixtures

In preparation for carrying out experiments to determine the effect of hydroxyl ion concentration on the thermal death rate of *Bacterium coli*, the first step was the preparation of various buffer mixtures. During the course of the investigation, several different buffer mixtures were tried in an attempt to find a set of mixtures covering the alkaline range which would be free from any toxic effects caused by substances in the mixtures. Palitzsch's borax-boric acid mixtures, Sörensen's borate-sodium hydroxid mixtures, Ringer's secondary sodium phosphate-sodium hydroxid mixtures, Clark and Lubs' primary potassium phosphate-sodium hydroxid mixtures, were made up according to the directions given by Clark (1925). Since the mixtures were standardized electrometrically, no special precautions were taken to obtain materials of absolute purity. The sodium bicarbonate-sodium hydroxid mixtures were made up by adding M/10 sodium hydroxid to M/15 sodium bicarbonate till the desired pH values were obtained. In adjusting the peptone to various hydroxyl ion concentrations, N/10 sodium hydroxid was added to 2 per cent peptone.

Methods of determining pH

The electrometric method of determining the pH was used as the standard method. A Type K Leeds and Northrup potentiometer was used for this purpose. It was found desirable to use a colorimetric method also in this work. Preliminary adjustments of the exposure media and check determinations before and after experiments were made with a colorimetric method. For this purpose a La Motte Hydrogen Ion Testing Outfit, Model B, was used. This method is a refined spot test in which small, shallow glass vials are used as containers for the substances to be tested, and these are placed on a porcelain slab. By comparison with the colors of standard buffer solutions in other glass vials, to which indicator has been added, the pH of an unknown is readily determined. Check determinations with the electrometric method showed that the results were accurate to within 0.2 pH. The indicators used were as follows:

NAME OF INDICATOR	pH RANGE	CONCENTRATION AND SOLVENT
Brom thymol blue	6.0–7.6	As described by Clark (1925).
Phenol red	6.8–8.4	
Cresol red	7.2–8.8	
Thymol blue	8.0–9.6	
Cresol phthalein	8.2–9.8	
Thymolphthalein	9.3–10.5	0.04 gram in 50 cc. alcohol and 50 cc. water
Tropaeolin O	11.1–12.7	0.04 gram in 100 cc. water
Sodium indigo disulfonate	11.4–14.0	0.10 gram in 100 cc. water

History of culture of bacterium coli used

The culture used was obtained from Dr. Levine of the Iowa State College, and according to his classification was culture No. 24. It gave typical *Bact. coli* reactions: Positive methyl red test, negative Voges-Proskauer test, and did not ferment sucrose. It was carried on agar slants for three months previous to this experiment. During the investigation the culture was transferred daily in 1 per cent peptone solution. These cultures were grown at room temperature which remained about 25°C.

Preliminary experiments

Cultures of *Bacterium coli* grown in nutrient broth five days at room temperature were used in the first 2 experiments. The original broth culture was diluted 10 times with sterile water and this diluted culture was plated out on standard agar and counts made after forty-eight hours' incubation of 37°C. One cubic centimeter of this suspension was inoculated into each tube of exposure medium, there being 9 cc. of medium in each tube. The pH values of the different media ranged from 7 to 13. The number of bacteria in each cubic centimeter of the suspension in

TABLE 1

Effect of pH of exposure medium on destruction of Bacterium coli by heat

Culture grown in nutrient broth 5 days at 25°C.

pH SUSPENSION	SUSPENSION EXPOSED TO TEMPERATURE OF 50°C.		SUSPENSION EXPOSED TO TEMPERATURE OF 55°C.		MEDIUM OF EXPOSURE
	At start	After 10 minutes	At start	After 10 minutes	
	number per cc.	*number per cc.*	*number per cc.*	*number per cc.*	
7.0	53,000,000	10,200,000	71,000,000	4,000	Borax-boric acid
8.0	53,000,000	1,920,000	71,000,000	0	Borax-boric acid
9.0	53,000,000	0	71,000,000	0	Borax-boric acid
10.0	53,000,000	0	71,000,000	0	Borate-NaOH
11.0	53,000,000	0	71,000,000	0	Na_2HPO_4-NaOH
12.0	53,000,000	0	71,000,000	0	Na_2HPO_4-NaOH
13.0	53,000,000	0	71,000,000	0	NaOH

the exposure tubes at the start was computed from the count of the diluted culture. After 10 minutes' exposure 1 cc. of the suspension in the exposure tube was plated out on standard agar. One experiment was made at 50°C. and the other at 55°C.

The results are recorded in table 1. It can be seen from the table that at 50°C. a pH of 9 or greater was necessary for complete destruction in ten minutes, while at pH 7 about a fifth of the original number of bacteria survived after ten minutes' exposure. At 55°C., however, the number of bacteria was reduced to a small fraction of 1 per cent of the original number after ten minutes' exposure at pH 7, and the culture was completely destroyed at pH 8.

Other experiments were carried out for the purpose of determining the effects of different buffer mixtures on the viability of *Bacterium coli*. The same experimental procedure as described above was followed here also. In tables 2 to 7, inclusive, the results of this group of experiments are recorded. The discussion

TABLE 2

Comparison of effects of different buffer mixtures on thermal death rate of Bacterium coli

Temperature of exposure 50°C. Culture grown in 1 per cent peptone 3 days at 25°C.

TIME	MEDIUM OF EXPOSURE				
	Water	Borax-boric acid pH 7.0	Phosphate-NaOH pH 7.0	Phosphate-NaOH pH 8.2	Glycine-NaOH pH 8.4
	number per cc.	*number per cc.*	*number per cc.*	*number per cc.*	*number per cc.*
At start	7,800,000	7,800,000	7,800,000	6,600,000	6,600,000
After five minutes				270	504,000
After ten minutes	1,900,000	337,000	2,930,000	5	5,880
After twenty minutes				0	31

TABLE 3

Effect of concentration of hydroxyl ions in destruction of Bacterium coli

Temperature of exposure 25°C. Culture grown in 1 per cent peptone 3 days at 25°C.

pH OF SUSPENSION	SUSPENSION AT 25°C.		MEDIUM OF EXPOSURE
	At start	After 10 minutes	
	number per cc.	*number per cc.*	
8.0	1,510,000	1,270,000	KH_2PO_4–NaOH
9.1	1,510,000	730,000	KH_2PO_4-NaOH
10.0	1,510,000	25,000	$NaHCO_5$-NaOH
11.1	1,510,000	0	Na_2HPO_4-NaOH
12.0	1,510,000	0	Na_2HPO_4-NaOH
13.0	1,510,000	0	Na_2HPO_4-NaOH

of these results is taken up under Discussion, but one or two observations at present are probably advisable. In table 2 it can be seen that the mixture containing boric acid, which is a mild disinfectant, is more toxic than the phosphate mixture covering the same pH range. How much of this difference is

due to the concentration of salts and how much is due to the toxicity of certain ions and molecules is difficult to say. Other factors besides these two may enter also. The data are presented to show the necessity of a medium which exerts a constant effect

TABLE 4

Effect of concentration of hydroxyl ions in destruction of Bacterium coli

Temperature of exposure 40°C. Culture grown in 1 per cent peptone 3 days at 25°C.

pH OF SUSPENSION	SUSPENSION AT 25°C.		MEDIUM OF EXPOSURE
	At start	After 10 minutes	
	number per cc.	*number per cc.*	
7.0	1,600,000	1,520,000	KH_2PO_4-NaOH
8.0	1,600,000	1,490,000	KH_2PO_4-NaOH
9.1	1,600,000	222,000	$NaHCO_3$-NaOH
10.0	1,600,000	400	Na_2HPO_4-NaOH
11.1	1,600,000	0	Na_2HPO_4-NaOH
12.0	1,600,000	0	Na_2HPO_4-NaOH
13.0	1,600,000	0	NaOH

TABLE 5

Effect of concentration of hydroxyl ions in destruction of Bacterium coli

Temperature of exposure 46°C. Culture grown in 1 per cent peptone 3 days at 25°C.

pH OF SUSPENSION	SUSPENSION AT 46°C.		MEDIUM OF EXPOSURE
	At start	After 10 minutes	
	number per cc.	*number per cc.*	
8.9	14,500,000	1,944,000	Glycine
9.1	14,500,000	1,296,000	Glycine
9.3	14,500,000	307,000	Glycine
9.5	14,500,000	21,600	Glycine
9.7	14,500,000	7	Glycine
9.9	14,500,000	0	Glycine
10.9	14,500,000	0	Na_2HPO_4-NaOH

throughout the pH range of the experiment. Unless such a medium is used it is difficult to make comparisons between the effects of different concentrations of hydroxyl ions. In the data presented in tables 2 to 7, inclusive, it can be seen that the higher

the concentration of hydroxyl ions the more effective is the destruction of bacteria by heat. Within the same buffer mixture the effects of different pH values can be compared even though the death rates in different mixtures at the same pH are not the same.

TABLE 6

Effect of pH of medium of exposure on thermal death rate of Bacterium coli

Temperature of exposure 50°C. Culture grown in 1 per cent peptone 3 days at 25°C.

pH OF SUSPENSION	SUSPENSION AT 50°C.				MEDIUM OF EXPOSURE
	At start	After 5 minutes	After 10 minutes	After 20 minutes	
	number per cc.	*number per cc.*	*number per cc.*	*number per cc.*	
7.0	6,600,000	49,700	2,300	190	KH_2PO_4-NaOH
7.5	6,600,000	13,300	190	40	KH_2PO_4-NaOH
7.9	6,600,000	210	0	0	KH_2PO_4-NaOH
8.2	6,600,000	270	5	0	KH_2PO_4-NaOH
8.4	6,600,000	504,000	5,880	31	Glycine
9.0	6,600,000	80	0	0	Glycine

TABLE 7

Effect of pH of medium of exposure on thermal death rate of Bacterium coli

Temperature of exposure 52°C. Culture grown in 1 per cent peptone 3 days at 25°C.

pH OF SUSPENSION	SUSPENSION AT 52°C.		MEDIUM OF EXPOSURE
	At start	After 10 minutes	
	number per cc.	*number per cc.*	
6.96	2,930,000	0	KH_2PO_4-NaOH
7.89	2,930,000	0	KH_2PO_4-NaOH
8.15	2,930,000	0	KH_2PO_4-NaOH

Results with peptone as exposure medium

A 2 per cent solution of Difco peptone was prepared, several portions adjusted with M/10 sodium hydroxid to the desired pH values, and each mixture diluted with water so that the sum of the sodium hydroxid and water added in each case was a constant amount. Precipitates were filtered out after the solutions had been heated once. The pH changed during sterilization, the more

alkaline solutions decreasing as much as 0.4 pH. Since the pH values of the exposure media were determined immediately after each experiment, the final pH was the only one of importance.

TABLE 8

Effect of pH of medium of exposure on thermal death rate of Bacterium coli

Temperature of exposure 50°C. Medium of exposure—peptone solution.

pH OF SUSPENSION	SUSPENSION AT START	SUSPENSION AFTER				
		3 minutes	6 minutes	10 minutes	15 minutes	20 minutes
	number per cc.					
6.95	2,400,000	+	+	+	+	+
7.36	2,400,000	+	+	+	+	+
7.87	2,400,000	+	+	+	+	+
8.28	2,400,000	+	+	+	+	+
8.78	2,400,000	+	+	+	+	+
9.24	2,400,000	+	+	+	−	−
9.66	2,400,000	+	+	−	−	−
10.09	2,400,000	+	−	−	−	−
10.38	2,400,000	+	−	−	−	−

+, suspension not destroyed; −, suspension destroyed.

TABLE 9

Effect of pH of medium of exposure on thermal death rate of Bacterium coli

Temperature of exposure 52°C. Medium of exposure —peptone solution

pH OF SUSPENSION	SUSPENSION AT START	SUSPENSION AFTER				
		2½ minutes	5 minutes	10 minutes	15 minutes	20 minutes
	number per cc.					
7.05	2,380,000	+	+	+	+	+
7.44	2,380,000	+	+	+	+	−
7.94	2,380,000	+	+	+	−	−
8.34	2,380,000	+	+	−	−	−
8.78	2,380,000	+	−	−	−	−
9.22	2,380,000	+	−	−	−	−
9.69	2,380,000	+	−	−	−	−
10.11	2,380,000	−	−	−	−	−
10.28	2,380,000	−	−	−	−	−

+, suspension not destroyed; −, suspension destroyed.

Determinations, made before and after the disinfection tests in several experiments, showed that the pH did not change appreciably during the experiment.

Instead of making plate counts as in previous experiments, a different procedure was followed in this group of experiments. In order to determine when all the bacteria had been destroyed, 0.1 cc. samples of the bacterial suspension were inoculated into tubes of 1 per cent peptone. These samples were drawn from the exposure tubes at various intervals during the time of exposure. The inoculated tubes of 1 per cent peptone were then incubated at 37°C. for twenty-four hours. Turbidity of the peptone medium was recorded as an indication of growth. In this way

TABLE 10

Effect of pH of medium of exposure on thermal death rate of bacterium coli

Temperature of exposure 54°C. Medium of exposure—peptone solution

pH OF SUSPENSION	SUSPENSION AT START	SUSPENSION AFTER				
		1 minute	2 minutes	5 minutes	10 minutes	20 minutes
	number per cc.					
7.0	3,920,000	+	+	+	+	−
7.4	3,920,000	+	+	+	+	−
7.9	3,920,000	+	+	+	−	−
8.3	3,920,000	+	+	+	−	−
8.6	3,920,000	+	+	−	−	−
9.0	3,920,000	+	+	−	−	−
9.5	3,920,000	+	+	−	−	−
9.8	3,920,000	+	−	−	−	−

+, suspension not destroyed; −, suspension destroyed.

times necessary to destroy all the bacteria at various pH values could be readily determined. Although the experimental error was large, many more points could be determined with this method than by the plating method. The results of these experiments are recorded in tables 8, 9, and 10. Curves plotted from these results are given under Discussion.

Determination of death rate of bacterium coli at 46°C. at different hydroxyl ion concentrations

Another series of experiments was carried out for the purpose of determining the death rate of *Bacterium coli* at various hydroxyl

ion concentrations. The procedure was altered somewhat from that used in any of the other experiments. A measuring flask (100 cc.) was used to hold the exposure medium. The flask was suspended in a constant temperature water bath. Five cubic centimeters of a three-day old culture, of which a sample was plated out at the time of inoculation, were inoculated into 50 cc. of the glycine-sodium hydroxid buffer mixture. The glycine-sodium hydroxid mixture as described by Clark (1925) was diluted to twice its volume. The exposure flask after the addition of the 5 cc. of the culture was shaken several times and was also shaken before the removal of each sample, but was at no time

TABLE 11

Death rate of Bacterium coli at pH 9.05

Temperature of exposure 46°C. Medium of exposure—glycine-NaOH mixture (diluted 1:1). Culture grown in 1 per cent peptone 3 days at 25°C.

TIME	SUSPENSION IN EXPOSURE FLASK AT 46°C.
minutes	*number per cc.*
0	27,400,000
2	28,300,000
4	33,300,000
8	28,500,000
12	29,000,000
16	25,500,000
20	19,100,000

taken from the water bath. At two-, four-, eight-, twelve-, sixteen-, and twenty-minute intervals 1.0 cc. samples were drawn and plated out on standard agar in duplicate. The plates were incubated at 37°C. for forty-eight hours.

From the data recorded in tables 11 to 13, inclusive, it is possible to determine the rate of death of *Bacterium coli* at 46°C. at 3 different hydroxyl ion concentrations. At pH 9.05 the decline in numbers was very slight even after twenty minutes' exposure. At a pH of 9.34 there was a significant increase in the death rate, and at a pH of 9.89 the decline in numbers was quite rapid. At the end of sixteen minutes' exposure at pH 9.89 all the bacteria

had been destroyed. The original concentrations of bacteria in all 3 experiments were approximately the same.

In tables 11 to 13, inclusive, one other thing of interest should be noted. If the logarithm of the number of survivors is plotted against time in minutes, a curve and not a straight line is obtained

TABLE 12

Death rate of Bacterium coli at pH 9.34

Temperature of exposure 46°C. Medium of exposure—glycine-NaOH mixture (diluted 1:1). Culture grown in 1 per cent peptone 3 days at 25°C.

TIME	SUSPENSION IN EXPOSURE FLASK AT 46°C.
minutes	*number per cc.*
0	30,000,000
2	28,200,000
4	28,200,000
8	11,500,000
12	4,940,00
16	610,000
20	90,000

TABLE 13

Death rate of Bacterium coli at pH 9.89

Temperature of exposure 46°C. Medium of exposure—glycine-NaOH mixture (diluted 1:1). Culture grown in 1 per cent peptone 3 days at 25°C.

TIME	SUSPENSION IN EXPOSURE FLASK AT 46°C.
minutes	*number per cc.*
0	34,300,000
2	15,800,000
4	1,160,000
8	9,000
12	100
16	0
20	0

in each instance. This fact shows that the death rate is not logarithmic. Velocity constants calculated by using the equation for the first order chemical reaction increased toward the end of the disinfection process. In these cases the rates of disinfection could hardly be measured satisfactorily by the velocity constants.

DISCUSSION OF RESULTS

pH-temperature relation

Figure 1 shows graphically the relation between the hydroxyl ion concentration and temperature in influencing the thermal death rate of *Bacterium coli*. The points plotted on this graph were taken from various experiments (see tables 3, 4, 5, 6, and 7)

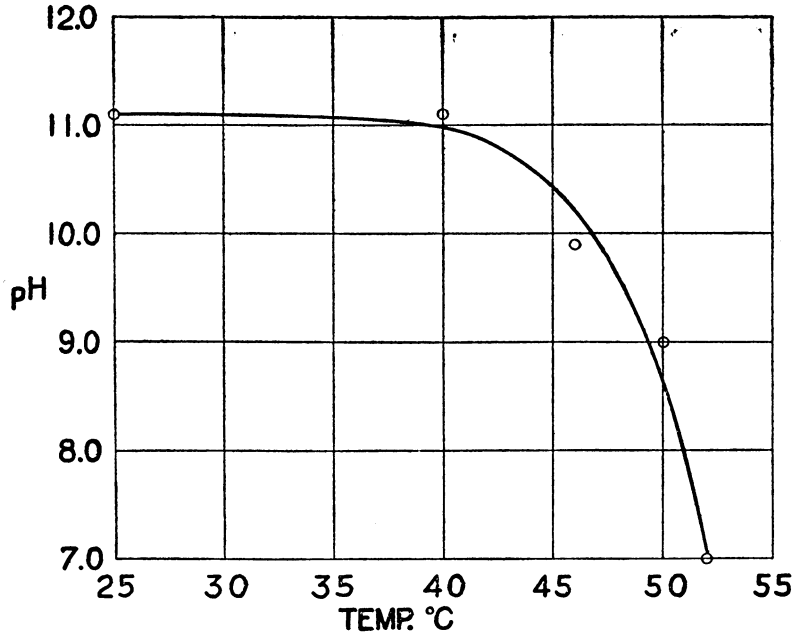

FIG. 1. pH-TEMPERATURE RELATION, SHOWING HYDROXYL ION CONCENTRATION NECESSARY FOR DESTRUCTION OF BACTERUIM COLI AT VARIOUS TEMPERATURES

Time of exposure ten minutes. Data from tables 3 to 7, inclusive

where the time of exposure was ten minutes. The points at 25° and 40° were obtained with secondary sodium phosphate buffer mixture as the medium of exposure; the points at 46° and 50° were obtained with the glycine buffer as the medium of exposure; and the point at 52° was obtained by the use of the primary potassium phosphate mixture. As has been shown above, the primary potassium phosphate mixture at approximately the same pH was

much more effective in the destruction of *Bacterium coli* than was the glycine mixture. Probably if the phosphate buffer had been diluted so as to decrease its buffer value, or if a more favorable medium such as glycine had been used, the point determined by a pH value of 7 would have fallen at a higher temperature. This would have made the curve slope more gradual at the higher temperature. However, the general relation is shown by the

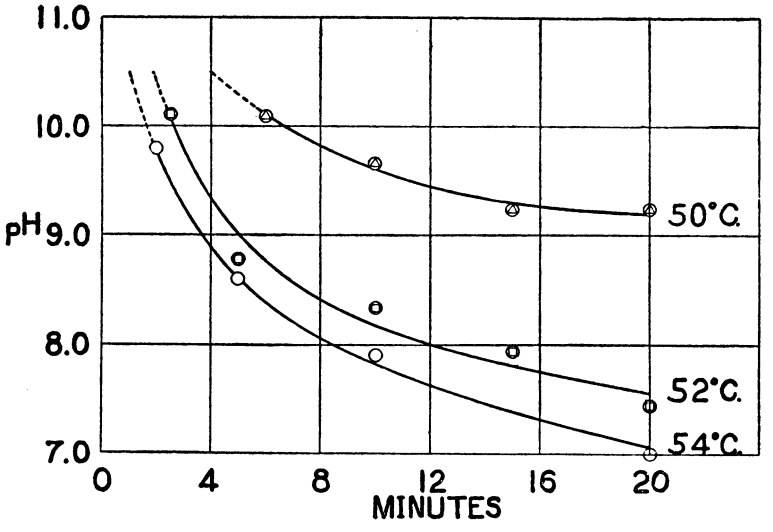

FIG. 2. pH-TIME RELATION, CURVES FOR 3 TEMPERATURES, SHOWING TIMES NECESSARY FOR DESTRUCTION OF BACTERIUM COLI AT VARIOUS pH VALUES

Medium of exposure: Peptone solution. Data obtained from tables 8 to 10, inclusive.

curve as it stands. Any point on the curve represents conditions which are destructive to *Bacterium coli* when the time of exposure is ten minutes.

pH-time relation

From the results of the experiments in which peptone, adjusted to the various pH values, was used as the medium of exposure, the curves in figure 2 were plotted; pH values on the ordinates, time in minutes on the abscissas. (For data see tables 8 to 10,

inclusive.) As is shown by the curves, when the temperature is decreased a higher pH value is necessary for destruction in a given length of time of exposure.

Temperature-time relation

From the curves in figure 2 the temperature and the time necessary for complete destruction of the bacteria at a given pH may be obtained. As shown in figure 3 a series of curves is

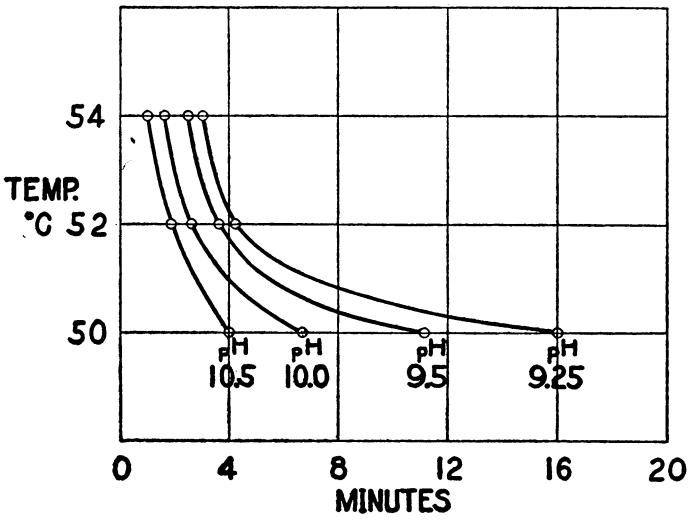

FIG. 3. TEMPERATURE-TIME RELATION, CURVES FOR 4 pH VALUES, SHOWING TIMES NECESSARY FOR DESTRUCTION OF BACTERIUM COLI AT VARIOUS TEMPERATURES

Data obtained from figure 2

obtained which show the relation of time to temperature at various pH values. The effect of the pH is quite evident again here, but one additional point is shown; namely, that a given difference in pH causes a greater difference in the thermal death time at 50° than at 54°. As the temperature is raised the curves tend to converge and approach zero time at high temperatures. As the temperature is lowered the curves flatten out, and the curves of the lower pH values extend to much longer times.

Similar relations for various organisms have been shown by Bigelow (1922). Weiss (1921) has determined similar curves with spores of *Clostridium botulinum* in which the temperature-time relation is shown at various pH values.

SUMMARY

1. Experiments have been made on the resistance of *Bacterium coli* to various conditions of temperature and hydroxyl ion concentration. Test mixtures used for this purpose were solutions of: Primary potassium phosphate-sodium hydroxid; borax-boric acid; sodium bicarbonate-sodium hydroxid; borate-sodium hydroxid; secondary sodium phosphate-sodium hydroxid; glycine-sodium hydroxid; peptone-sodium hydroxid; and sodium hydroxid alone.

2. It has been found that different buffer mixtures of approximately the same pH value exert very different germicidal effects. The effects of pH cannot be compared when 2 or more different buffer systems are used, but can be compared within a single buffer system.

3. Increase in the pH on the alkaline side of neutrality of a given solution has been shown to increase its power to destroy *Bacterium coli* at a given temperature.

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