STUDIES ON SALT ACTION

VIII. THE INFLUENCE OF CALCIUM AND SODIUM SALTS AT VARIOUS HYDROGEN ION CONCENTRATIONS UPON THE VIABILITY OF BACTERIUM COLI

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OBJECTIVES

In an earlier paper (Falk, 1923) our present state of knowledge in regard to the physiological influence of mineral salts upon bacteria has been somewhat comprehensively reviewed. The experiments here reported were designed to determine with some exactness the effect upon bacterial viability of sodium and calcium chlorides, alone and in combination. Our work has extended over a long period, the first preliminary communications (Winslow and Falk, 1918) having been made over four years ago. The early experiments at times yielded conflicting results and it was only by a careful study of the hydrogen ion factor (Falk, 1920) that we succeeded in explaining these inconsistencies along the lines since so well worked out for problems of bacterial growth by Holm and Sherman (1921) and Sherman and Holm (1922). The study presented here deals with the viability of *Bacterium coli* in distilled water, in various concentrations of NaCl, and in various concentrations of $CaCl_2$ with varying hydrogen ion concentrations for each solution; while a following paper (IX) deals with viability in various mixtures of the two salts mentioned above.

TECHNIQUE

All of the experiments here reported were conducted with a single strain of *Bact. coli* which was isolated from a polluted stream near New Haven in the autumn of 1916 and has been

used ever since for various studies of viability conducted in this laboratory.

The bacteria needed for each experiment were cultivated on standard nutrient agar slants, or Petri plates or in Kolle flasks at 37°C. for sixteen to twenty-four hours. The growth from the surface was washed off in water or in the appropriate salt solution, shaken for three to five minutes to break up clumps, filtered through paper or absorbent cotton and then added in 1 cc. portions to the bottles of sterilized water or salt solution in which the viability was to be tested, the solutions having previously been warmed to a temperature of 37°. Quite uniformly the concentration of bacteria at the beginning of each test was 20,000,000 to 40,000,000 per cubic centimeter. Only occasionally was it somewhat lower or higher. Plates were made on agar one minute after seeding to determine the initial number present. The bottles were then replaced in the incubator at 37° and removed for the estimation of the number of surviving organisms at various desired intervals. All counts were made on a standard nutrient agar after incubation for twenty-four hours at 37°C.

The water used in these experiments was carefully distilled from a Barnstead still, or was twice redistilled in glass vessels and again redistilled from glass and into a block tin condenser. Whenever tested it gave a negative test for ammonia with Nessler's reagent. The hydrogen ion concentration of the water used in the earlier experiments was generally between pH 6.4 and 6.8 but was not recorded in regular routine. In certain other tests the initial acidity of the water was higher, usually between pH 5.2 and 5.8.

On the addition of the bacterial cells this reaction would be shifted to a pH of 8.0 or above by the alkaline dissociation products of the suspension. Later there was a tendency for the reaction to return to a zone of hydrogen ion concentration between pH 7.0 and pH 7.4, after an incubation of from one to forty-eight hours. The effect of initial reaction is however often an important one; and in our later work this factor has been carefully controlled.

In those experiments in which we recorded the pH of the solutions, the measurements of acidity were made by the usual

colorimetric procedure, either before or after the addition of the suspension of bacteria according as the pH measurement value "before" or "after" seeding was desired. When the pH of a solution was readjusted, great pains were taken to introduce as few new ions and in as small a concentration as reasonably rapid work permitted. In adjusting the pH of bacterial suspensions in water and in sodium chloride solutions, HCl and NaOH were used. With calcium chloride solutions HCl and $Ca(OH)_2$ were used. The additions of acid or alkali were made by measuring the pH of an aliquot from the test solution, consulting a titration curve of a similar prepared solution to determine the quantity of acid or alkali to be added to give the desired pH and reading the pH of another aliquot after the appropriate addition. Excepting for the water suspensions, the addition of these reagents involved the introduction of no new ions. Because of the comparatively low buffer content of the suspensions in water or in dilute aqueous solutions of these salts, the amounts of acid or alkali added to give the stated pH values were exceedingly minute and the increase in concentration of Na+ and Ca++ ions in the salt solutions incidental to these additions was generally entirely negligible by comparison with the concentration of these ions already present.

Following the work of Cohen (1922) we made some studies of viability in dilute (M/500) buffer solutions. This particular group of studies was carried only far enough to indicate that the buffers, even in such dilution, were producing effects specific to themselves. The results of these experiments are therefore omitted from this paper on the viability of *Bact. coli* in pure water or in solutions of single salts. The study of pH influences upon viability in the absence of buffers is much more difficult and the results from individual experiments are more uncertain than in their presence, but it makes possible observations of certain phenomena which it would be otherwise impossible to observe.

The sodium chloride used in our earlier investigations was purified by treatment with calcium hydroxide (to remove magnesium) and with sodium carbonate (to remove calcium and barium), and was then recrystallized from pure concentrated

hydrochloric acid and from pure water; the calcium chloride was twice recrystallized and the crystals dried in a current of air and in the drying oven. In our later experiments we found that "chemically pure" (analyzed) commercial preparations of these salts were entirely satisfactory when the limiting concentrations of impurities were low.

VIABILITY IN DISTILLED WATER

First of all it was important to determine the behavior of the particular strain of *Bact. coli* used in our experiments when suspended in distilled water free from the presence of notable amounts of mineral salts. An earlier investigation of the viability of this strain by Winslow and Cohen (1918) had shown that it suffered only a slight reduction in numbers when suspended in water for twenty-four hours (82 per cent surviving) but that after this period it gradually died off, only 49 per cent remaining after three days and only 5 per cent after five days.

We ourselves conducted 37 different experiments of this kind during the first years of this study, the results of which are presented in table 1. The pH values at the head of each column indicate the acidity to which the solutions were adjusted immediately after seeding the bottles of water with the bacterial suspensions and at which they were maintained by readjustment of reaction at each observation time if they showed a variation of 0.2 or more from the stated pH. A uniform tendency for the pH to shift towards the zone 7.0 to 7.4 is found here as well as in the experiments with suspensions of *Bact. coli* in sodium and calcium chloride solutions. This point will be treated more fully later. Tests under the column headed "x" were early experiments conducted in solutions, initially alkaline, but in which the pH was neither recorded nor readjusted.

The figures given for the per cent of organisms surviving at each period represent in each case the average of from 2 to 11 tests conducted under the conditions specified, the first seven columns including tests made at known pH values, the last column the earlier tests in which hydrogen-ion concentrations were not recorded. In this and succeeding tables a heavy rule

has been drawn to indicate the range of time and hydrogen-ion concentration most nearly corresponding to a one third destruction of bacteria. There are of course occasional irregularities in the viability curve which make the establishment of this one-third reduction point somewhat conjectural in certain instances as in the columns of table 1 for pH values of 5.0 and 6.0.

It appears from table 1 that at pH 6.0 the reduction in the number of bacteria is very slight, while at pH 5.0 it is somewhat greater; in more acid or more alkaline solutions the viability decreases rapidly. This table, as indicated in its lowest line,

TABLE 1

Viability of Bact. coli in distilled water

HOURS	PER CENT ALIVE AT pH							
	4.0	5.0	6.0	6.5	7.0	7.5	8.0	x
1	87	88	84	92	68	77	79	196
3	39	71	74	66	54	24	52	134
6	4	48	64	30	24	8	12	78
9	1	68	82	7	17	5	12	89
24	0	6	77	2	23	3	10	57
Number of experiments...	2	2	4	2	10	2	4	11

includes the averages of 37 experiments; for the 9-hour period we conducted a more extensive series of tests, this period being chosen because our earlier work had indicated that the physiological influences which we were desirous of measuring were most clearly evident at that time. In table 2 we have collected all of our nine hour data, including those cited in table 1, to give a final table which represents the results of seventy-nine tests.

The curve which expresses the relation between pH and the viability of *Bact. coli* in distilled water is obviously smooth and indicates the existence of a zone of physiological tolerance be-

tween 5.0 and 7.0 which is exceeded more abruptly at the acidic than at the alkaline extreme.

There is some discrepancy between the results of the first 37 tests (table 1) and those of the whole 79 tests (table 2), as would naturally be expected in dealing with a problem of such complexity as the viability of bacteria. Only at pH 7.0 however is this discrepancy serious, the first group of tests showing a 17 per cent survival and the entire series a 54 per cent survival. The results in general confirm the earlier studies of Winslow and Cohen (1918) and indicate that the strain of *Bact. coli* studied persists in distilled water for twenty-four hours with only slightly diminished numbers at a pH of 6.0, but that it shows a sharp reduction in solutions which are more acid than 5.0 or more alkaline than 7.0.

TABLE 2

Viability of Bact. coli in distilled water after nine hours

	pH						
	4.0	5.0	6.0	7.0	7.5	8.0	x
Per cent of bacteria surviving.....	1	82	106	54	35	12	89

It will be noted that the viability for unregulated (and unknown) pH values (in the column headed "x") corresponds approximately to the results obtained at a regulated pH of 6.0, showing little or no decrease in 9 hours. We have reason to believe that the solutions used in these early tests were initially distinctly alkaline (following the addition of bacteria), with a pH in the neighbourhood of 8.0, which would have been expected to show a marked reduction in numbers. The fact that an unregulated alkaline solution gives the same results as a regulated neutral solution, as will be explained in a succeeding paragraph, is due to the buffering properties of the bacterial cells or of their products in solutions whose hydrogen-ion concentrations are not artificially controlled.

Our next problem was to determine the influence exerted upon the viability of our test organism by a salt with a monovalent and one with a bivalent cation. For this purpose we chose the chlorides of sodium and calcium.

THE EFFECT OF SODIUM CHLORIDE IN DIFFERENT CONCENTRATIONS AND AT VARIOUS ACIDITIES

To study the influence of sodium chloride upon the viability of *Bact. coli* we suspended the organisms in salt solutions of varying strengths, from 0.0145 molar (0.1 isotonic) to 1.45 molar (10 isotonic). The technique of manipulation was otherwise the same as that previously described. At the beginning of our work we made up our solutions in fractions and multiples of isotonic strength, following the example of Loeb and other students of the higher organisms. It seems doubtful, however, how far the conception of isotonicity applies to the bacterial cell; and in order to avoid any a priori assumptions we have expressed our data in the present paper in molar terms. For comparability we have, however, kept the same actual concentrations throughout, in spite of the fact that when expressed in molar terms the figures appear as irregular fractions.

Tables 3 to 8 present our findings from 99 experiments in which various concentrations of sodium chloride were studied through twenty-four hour periods under different pH conditions. The figures in table 3 for 0.0145 and 0.0725 molar solutions of sodium chloride show an unquestionably favorable effect on viability. By comparison with the data in tables 1 and 2 which show the mortality in distilled water it is seen that after any incubation period the per cent of bacteria surviving is greater in these salt solutions than in water even at the most favorable pH (6.0). Similarly, table 4 shows that viability is favored in 0.145 molar sodium chloride solution. Even in the four test solutions incubated at pH 5.0 to 6.0 there were still 100 or more per cent of the bacteria alive after 9 or 24 hours as compared with 82 to 77 per cent alive at the favorable pH 6.0 in water. On the other hand a 0.435 molar NaCl solution in one experiment (table 5) conducted at pH 7.3–7.6, was distinctly unfavorable for the survival of *Bact. coli*, and in six experiments in which pH was not regulated a very slight toxicity was manifest, the reduction in numbers, however, being scarcely greater than in distilled water with unregulated reaction. The averages of these 7

TABLE 3

Viability of Bact. coli in sodium chloride solution; 0.1 isotonic (0.0145 M) and 0.5 isotonic (0.0725 M)

HOURS	PER CENT ALIVE	
	0.0145 M—pH not known	0.0725 M—1 experiment pH not known, 1 experiment pH 7.0
1	100	91
3	105	96
6	77	121
9	115	86
24	140	82
Number of experiments.....	7	2

TABLE 4

Viability of Bact. coli in sodium chloride solution; 1.0 isotonic (0.145 M)

HOURS	PER CENT ALIVE			
	ph 5.0—6.0	pH 7.0—7.4	pH not known	All pH values
1	75	90	97*	88*
3	85	91	105	97
6	81	71	101	91
9	101	76	83*	82*
24	114	80	89	84
Number of experiments.....	4	2	10	16

* One unusually high figure omitted from average.

TABLE 5

Viability of Bact. coli in sodium chloride solution; 3 isotonic (0.435 M)

HOURS	PER CENT ALIVE		
	pH 7.3—7.6	pH not known	All pH values
1	94	109	107
3	57	68	66
6	7	71	61
9	0.4	64	55
24	0	63	54
Number of experiments.....	1	6	7

TABLE 6

Viability of Bact. coli in sodium chloride solution; 5 isotonic (0.725 M)

HOURS	PER CENT ALIVE AT pH							
	4.0	5.0	6.0	6.5	7.0	7.5	8.0	Unknown
1	0+	6	82	101	94	98	67	85
3	0+	0.4	78	72	71	38	52	67
6		0.1	54	24	57	26	21	49
9		0.2	43	12	33	11	9	46
24		0.1	13	1	5	0+	0+	30
Number of experiments..	1	4	6	2	10	3	3	18

TABLE 7

Viability of Bact. coli in sodium chloride solution; 6 isotonic (0.870 M)

HOURS	PER CENT ALIVE AT pH				
	6.0	6.5	7.0	7.5	8.0
1	86	86	23	54	55
3	15	35	1	6	6
6	11	6	0.2	0.4	0.2
9	1	1	0+	0+	0+
24	0+	0+	0+	0+	0+
Number of experiments..	A single experiment				

TABLE 8

Viability of Bact. coli in sodium chloride solution; 7 isotonic (1.015 M) and 10 isotonic (1.450 M)

HOURS	PER CENT ALIVE—pH NOT KNOWN	
	1.015 M	1.450 M
1	91	61
3	51	16
6	33	1
9	20	0+
24	8	0+
Number of experiments.....	9	10

experiments indicate that sodium chloride is probably slightly toxic in a 0.435 molar concentration. From the results of 47 experiments with sodium chloride in 0.725 M concentrations summarized in table 6 it appears that a fairly marked toxicity has been attained. When sodium chloride solution exceed 0.725 M concentrations the toxicites of the solutions increase rapidly. In the single experiment conducted with 0.870 molar concentration (table 7) the greatest viability was evidenced at pH 6.0 and 6.5 although the rate of mortality was at all points very great. Concentrations of 1.015 M and 1.450 M (table 8) are uniformly of marked toxicity, killing 80 to 100 per cent of the bacteria in nine hours (table 8).

Throughout all of this work we have found that when measuring viability in non-toxic concentrations of NaCl or in water an occasional test solution will show a decided toxicity not manifested by others of similar strength. Such variations are lost sight of when the data are tabulated in terms of averages as we have done. Lack of space forbids presentation of the individual findings in the hundreds of tests which we have conducted or even a brief discussion of the hypotheses upon which these experimental divagations may be explained. Fantus and Rumry (1920) have demonstrated that neither concentration of inoculum nor presence or absence of clumps is responsible for such variations. With solutions of molar strength the factor of variability appear to be almost completely abolished. In these concentrations NaCl is uniformly and markedly toxic. In general it seems to us that our experiments are sufficiently numerous to warrant the general conclusion that a 0.14 M or less concentration of sodium chloride is favorable to the survival of *Bact. coli;* that a 0.435 M concentration is without marked toxic action upon the bacterium at all pH values studied; that 0.725 M sodium chloride possesses a distinct harmful effect which is least marked in the zone pH = 6.0 to 7.0; and that solutions over a molar strength are powerfully toxic.

Inasmuch as the 99 tests with NaCl which are summarized in tables 3 to 8 were conducted over a period of about three and one-half years, we thought it wise to repeat the series in a single

experiment, in order to determine how accurately we could check the outstanding findings on the relation between concentration and toxicity of this earlier work. We seeded 40 test solutions from a single suspension of *Bact. coli* nineteen hours old in distilled water, kept in an ice bath during the seeding period. The solutions had been sterilized by boiling, instead of in the autoclave, to avoid contamination with ammonia and other volatile substances commonly present in the steam of sterilizers. Before seeding, the solutions had pH values of 8.4 to 8.6, and immediately after seeding with about 35,000,000 *Bact. coli*

TABLE 9

Viability of Bact. coli in various concentrations of sodium chloride

SOLUTION—CONCENTRATION NaCl	SURVIVAL OF BACTERIA AFTER NINE HOURS					
	Experiment 98		All previous experiments		All experiments	
	Per cent alive	Number of tests	Per cent alive	Number of tests	Per cent alive	Number of tests
0	95	5	89	11	91	16
0.0145 M	121	5	115	7	118	12
0.0725 M			86*	2	86*	2
0.145 M	93	5	83	10	86	15
0.435 M	70	5	64	6	67	11
0.725 M	56	5	46	18	48	23
0.870 M	33	5			33	5
1.015 M	18	5	20	9	19	14
1.305 M	6	5			6	5
1.450 M			1	10	1	10

* This figure is introduced to make the tabulation complete. Inasmuch as it is the average of only two tests its validity is perhaps questionable.

per cubic centimeter, of 8.4 to 8.7. For each concentration of sodium chloride there were 5 test solutions. The results of this experiment (Experiment 98) are summarized in table 9 where they are compared with the summarized data of all comparable previous experiments (64 in number) in which viability in sodium chloride was tested and in which there were no adjustments or readjustments of pH.

The unusually close checks obtained renewed our confidence in the accuracy of the results obtained. We may safely conclude that the summarizing column "All experiments" in table 9

describes with reasonable accuracy the viability of *Bact. coli* in solutions of sodium chloride of varying concentration in which no adjustment of pH is made during the course of the test.

When we compare these results, shown in table 9, obtained at unregulated and unknown pH values (but with solutions starting at a pH in the neighborhood of 8.0 or over) with those obtained at a pH value adjusted during the course of the experiment, we find that as with distilled water the unadjusted alkaline solutions give results comparable with those obtained at a regulated pH of about 6.0, (see tables 4, 5, and 6).

EFFECT OF CALCIUM CHLORIDE IN DIFFERENT CONCENTRATIONS AND AT VARIOUS ACIDITIES

Table 10 shows the results obtained with $CaCl_2$ solutions of less than M/10 strength. With the three more dilute solutions (0.00145 M 0.0145 M, 0.029 M) there was the usual variation between parallel bottles always manifest in non-toxic solutions, one bottle showing a decrease, while its companion showed a marked increase. The average results for all the concentrations mentioned, however, show a distinctly higher viability than is manifest in distilled water (96 per cent or more alive after twenty-four hours). These low concentrations, as in the case of NaCl seem to be definitely favorable to viability.

A concentration of 0.072 M $CaCl_2$ on the other hand seems to show toxic action; and with a solution of 0.145 M strength we found an even clearer toxicity, at least *in those solutions in which the pH was not measured or controlled*, (last column of table 11). The bacteria hold their own for the first hour but after that decrease rapidly. After two to three hours only 4 bottles out of 19 in one group, for example, contained over 15 per cent of their original numbers and after twenty-four hours 15 out of 18 bottles showed 1 per cent or less of their original germ content, while the other 3 showed 3 per cent, 10 per cent, and 36 per cent, respectively. For a total of 32 tests at unregulated pH we found 70, 40, 27, 22 and 16 per cent alive after one, three, six, nine and twenty-four hours, respectively. The results obtained with adjusted acidities will be discussed in a succeeding paragraph.

Concentrations of calcium chloride of 0.435 M strength and over showed at all reactions a still more rapid and uniform toxic effect than that indicated for the 0.145 M solution in table 11. Nine tests were made with 0.435 M solutions (table 12). In 6 out of the 9 less than 1 per cent of the bacteria remained after three

TABLE 10

Viability of Bact. coli in calcium chloride solutions; 0.01 isotonic (0.00145 M), 0.1 isotonic (0.0145 M), 0.2 isotonic (0.029 M), 0.5 isotonic (0.072 M)

HOURS	PER CENT ALIVE—pH NOT KNOWN			
	0.00145 M	0.0145 M	0.029 M	0.072 M
1		73	94	88
3	92	84	89	51
6	158	64	105	41
9	166	70	92	45
24	152	96	110	37
Number of experiments.....	2	20	3	5

TABLE 11

Viability of Bact. coli in calcium chloride solutions: 1 isotonic (0.145 M)

HOURS	PER CENT ALIVE AT pH							
	4.0	5.0	6.0	6.5	7.0	7.5	8.0	Unknown
1	42	79	120	127	84	70	86	70
3	1	9	120	108	75	87	99	40
6	0+	2	124	146	66	58	38	27
9	0+	1	78	109	71	61	31	22
24	0	0+	90	61	49	31	12	16
Number of experiments...	1	1	3	2	9	2	2	32

Buffered solutions not included.

hours (the other 3 bottles showing a survival of 19 per cent, 32 per cent and 28 per cent at this period). After six hours these 3 bottles in which the resistance was relatively high showed 2 per cent, 7 per cent and 3 per cent respectively and after nine hours, 0.1 per cent, 1.9 per cent and 0.2 per cent.

Ten tests were made in 0.725 M solutions with the following results. In 5 bottles less than 1 per cent of the bacteria survived after three hours (the per cent surviving in the other 5 bottles being 19 per cent, 3 per cent, 4 per cent, 4 per cent and 9 per cent, respectively). After six hours all had dropped to 1 per cent or less. Five tests in 1.015 M calcium chloride showed less than 1 per cent surviving after three hours in 4 bottles, 1 per cent and 4 per cent in the other 2 cases (table 12).

TABLE 12

Viability of Bact. coli in calcium chloride solutions: 3 isotonic (0.435 M), 5 isotonic (0.725 M), 7 isotonic (1.015 M), 10 isotonic (1.450 M)

HOURS	PER CENT ALIVE—pH NOT KNOWN				
	0.435 M		0.725 M	1.015 M	1.45 M
	pH=7.6				
1	64	37	25	0+	15
3	28	9	4	0+	2
6	3	1	0.4	0	0+
9	0.2	0+	0+	0	0+
24	0	0+	0+	0	0
Number of experiments..	1	9*	10	5	6

* Includes one experiment with pH unknown.

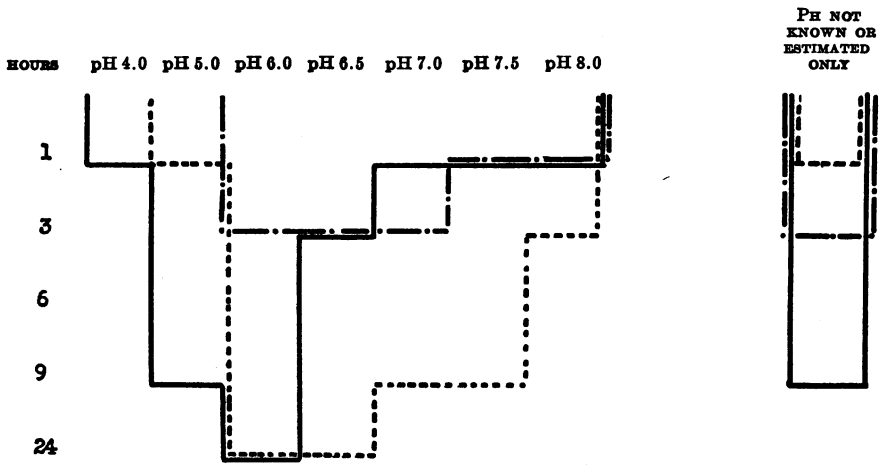
EFFECT OF REACTION IN THE PRESENCE OF SODIUM AND CALCIUM SALTS, RESPECTIVELY

We come now to a consideration of the somewhat puzzling results indicated by table 11 in regard to the influence of the reaction of the calcium chloride solutions upon viability. It has been pointed out above that, in distilled water and in the presence of NaCl, unregulated alkaline solutions (starting in the neighborhood of pH 8.0) show a high degree of viability similar to that obtained at an adjusted reaction of about pH 6.0; and this has been explained as probably due to reversion to a neutral reaction taking place in the presence of the bacterial cells. Yet with $CaCl_2$ the results observed in an unadjusted alkaline solution instead of corresponding with those obtained at a regulated pH of 6.0 show a high toxicity comparable with that characteristic of a regulated pH 8.0.

Tables 13 and 14 show very clearly the apparent conflict in the results observed. These tables include a number of special experiments conducted to cover the nine hour period alone in

TABLE 13

The relation between viability of Bact. coli and pH in distilled water, 5.0 isotonic NaCl (0.725 M) and 1.0 isotonic $CaCl_2$ (0.145 M) solutions



Lines indicate times and reactions at which one-third of the bacteria were dead.
——— Viability in distilled water.
.—. — Viability in 5.0 isotonic NaCl.
..... Viability in 1.0 isotonic $CaCl_2$.

TABLE 14

Effect of pH upon viability of Bact. coli in water, 5.0 isotonic (0.725 M) NaCl and isotonic (0.145 M) $CaCl_2$

SOLUTION	PER CENT OF BACTERIA SURVIVING AFTER NINE HOURS						
	5.0	5.5	6.0	7.0	7.5	8.0	x
Water	82		106	54	35	12	89
5.0 isotonic NaCl	27	20	87	76	8	9	46
1.0 isotonic $CaCl_2$	134		128	106	44	31	22

order to eliminate chance variations by obtaining averages based on a large number of observations. In these tables we have omitted all determinations not based on at least four tests. The results

differ at certain points from those presented in tables 1, 6 and 11 but where this is the case the results in the latter tables were based on 1 or 2 tests only. The bases for tables 13 (136 tests) and 14 (about 250 tests) are sufficiently broad to be reasonably reliable. In water and NaCl solutions the results obtained at an unadjusted alkaline pH correspond approximately to those observed at an adjusted pH of 6.0 or 7.0. In $CaCl_2$ solutions, on the other hand, the results obtained at an unadjusted alkaline pH correspond to those observed at an adjusted pH of about 8.0. Thus at an adjusted pH of 6.0, or at an adjusted pH of 8.0 or at any other adjusted pH, 0.725 M NaCl is slightly more toxic than

TABLE 15

Viability of Bact. coli in 0.145 M calcium chloride with hydrogen ion concentration increased by carbon dioxide from the breath

INCUBATION PERIOD	AVERAGE PER CENT SURVIVING	
	pH decreased by CO_2 from breath (average of 7 tests)	No CO_2 (average of 32 tests—table 12)
hours		
1	139	70
3	114	40
6	75	27
9	67	22
24	52	16
120	3	

0.145 M $CaCl_2$, in an unadjusted alkaline solution on the other hand 0.145 M $CaCl_2$ is distinctly more toxic than 0.725 M NaCl.

This particular concentration of $CaCl_2$ (0.145 M) seems then to display its characteristic toxicity only in an unregulated alkaline solution. The importance of the reaction factor was strikingly called to our attention by the high degree of viability manifest in certain tests in which the suspensions were stirred by blowing into them through the sampling pipettes. Table 15 shows how the carbon dioxide introduced in this way tended to abolish the toxic effect of 0.145 M $CaCl_2$.

An explanation of these phenomena has apparently been found by following out the observations made two years ago (Falk, 1920) as to the power of bacterial suspensions to regulate the pH

of their menstruum and the influence of salts upon that power. These earlier results warranted the assumption that in our experiments in which pH was uncontrolled the initial pH (in each case, an alkaline one) had remained alkaline in the $CaCl_2$ solutions but had shifted towards the neutral point (7.2) in the water and NaCl solutions. We tested this hypothesis in a series of 5 experiments (93 to 98) in which 50 test solutions were studied. We have summarized these results, which have been already reported in preliminary fashion (Winslow and Falk, 1922) in table 16.

It appears that at a pH controlled at about 6.0 neither water nor 0.145 M $CaCl_2$ has toxic action; that in water with an initial pH over 9.0 the reaction falls to about 8.0 in nine hours and no toxic effect is manifest; and, finally, that in a 0.145 M $CaCl_2$ solution of an initial pH over 9.0 the reaction changes but

TABLE 16

Change in pH and viability of Bact. coli in water and 1.0 isotonic (0.145 M) $CaCl_2$

SOLUTIONS	DISTILLED WATER			ISOTONIC $CaCl_2$ (0.145 M)		
	pH initial	After nine hours	Per cent bacteria alive after nine hours	pH initial	After nine hours	Per cent bacteria alive after nine hours
Unadjusted pH.....	9.2	8.0	91	9.2	8.9	1
Continuously adjusted pH.....	6.0	6.1	76	6.0	6.5	90

slightly and the bacteria show the high mortality to be expected in such an alkaline solution. It therefore appeared to us that the apparent conflict between the toxicities, absolute and relative, of the test solutions when pH was controlled and when it was uncontrolled, was explicable on the ground that although the unregulated water and sodium solutions on the one hand and calcium solutions on the other may begin an incubation period at the same pH, they do not have the same pH throughout the period. And, hence, the toxicities which are found in such solutions are not comparable with the relative toxicites of solutions kept by careful readjustment at a constant pH.

There remained the question whether the difference in reaction changes was quantitatively adequate to account for the observed differences in viability. The alkalinity of the unadjusted water

solution falls considerably it is true, but does it fall enough to account for the high viability displayed? Thus it appears in table 16 that a pH of 8.0 after nine hours (initial pH = 9.2) is non-toxic while, as previously indicated, a pH *maintained* at 8.0 throughout an experiment is highly toxic. It occurred to us that we might explain this apparent anomaly on the assumption that the regulating action of the bacteria operates in zones immediately surrounding the bacterial cells much more effectively than in the main bulk of the suspending fluid. If therefore the fluid is not stirred frequently or vigorously the bacteria in a solution which they are thus regulating may actually be exposed to a reaction much nearer neutrality than is indicated by a pH reading of the test solution as a whole. In such a case our pH readings do not represent the actual concentration of hydrogen-ions about the cell as they do in a suspension in which reaction is being controlled artificially by repeated shaking, examination of aliquot samples and readjustment of pH.

If this explanation is correct, it should follow that in bacterial suspensions whose pH is not being controlled by readjustments the shift in general pH should be greater in stirred than in unstirred bottles. That is, if in an alkaline suspension the bacteria secrete acidic substances (acidic relative to the pH of the suspensions) the pH of the suspension as a whole will remain comparatively unchanged if for lack of stirring of the solution these acidic substances remain in immediate contact with the bacteria, on the assumption that these acidic substances are secreted only as long as the menstrua are alkaline in the zone about the cell. On the other hand, if the solutions are stirred frequently the neutralizing substances secreted by the cells into the alkaline menstruum will be evenly distributed throughout the solution; the bacteria will be repeatedly exposed to a comparatively alkaline reaction and thus should be induced to secrete more and more of the neutralizing substances.

In order to test this assumption we prepared six bottles of each of the following solutions—water; 0.01, 0.1, 1.0, 3.0, 5.0, 7.0 and 10.0 isotonic NaCl; and 0.01, 0.1, 0.5, 1.0 and 3.0 isotonic $CaCl_2$ (isotonic solution is 0.145 M). All the bottles were set out

on a table where they would not be disturbed and were seeded from a single suspension of *Bact. coli* in distilled water so that each would contain approximately 30,000,000 bacteria per cubic centimeter. The pH of each was read after seeding and adjusted, if necessary, to 8.5 to 8.7. Each group of 6 bottles was then separated by random choice into two groups of 3 bottles each, one group for the "shaken" and the other for the "unshaken" tests. After three, six, nine and twenty-four hours' incubation,

TABLE 17

Effect of mechanical agitation upon change in pH. Suspensions of Bact. coli in water and in NaCl and $CaCl_2$ solutions

SOLUTION—MOLAR CONCENTRATION*	pH CHANGE (INITIAL pH = 8.5—8.7) AFTER INCUBATION FOR							
	Three hours		Six hours		Nine hours		Twenty-four hour	
	Shaken	Not shaken	Shaken	Not shaken	Shaken	Not shaken	Shaken	Not shaken
Water	0.2	0.0	0.9	0.1	1.2	0.2	1.3	0.8
NaCl								
0.00145	1.1	0.7	1.1	1.0	1.2	1.1	1.2	1.2
0.0145	1.1	0.5	1.2	1.2	1.3	1.3	1.3	1.3
0.145	0.4	0.2	1.2	0.9	1.3	1.0	1.2	1.1
0.435	0.3	0.0	1.0	0.1	1.1	0.3	1.2	0.9
0.725	0.3	0.0	1.1	0.1	1.0	0.2	1.1	0.9
1.015	0.7	0.0	0.9	0.1	0.9	0.2	1.2	0.8
1.450	0.0	0.0	0.0	0.0	0.2	0.2	0.9	0.5
$CaCl_2$								
0.00145	0.3	0.0	0.3	0.1	0.4	0.2	1.3	0.5
0.0145	0.4	0.2	0.7	0.2	0.9	0.3	1.2	0.6
0.0725	0.1	0.0	0.6	0.0	0.6	0.1	0.6	0.6
0.145	0.1	0.1	0.2	0.2	0.5	0.2	0.5	0.2
0.435	0.1	0.0	0.2	0.0	0.2	0.0	0.2	0.1

* 1 tonicity = 0.145 M.

pH samples were taken from each bottle. On these occasions, the "shaken" bottles were stirred vigorously before an aliquot was withdrawn. (They were also stirred at one-half hour intervals during the first nine hours between pH readings.) The "unshaken" bottles were never handled and were watched with great care to avoid accidental disturbance. Fine-tipped pipettes were used, and the samples were withdrawn slowly and carefully to avoid stirring and backwash. All of the test solutions were

allowed to become saturated with air before the experiment was begun. For the sake of convenience in manipulation, the work was done at room instead of incubator temperature. The findings are summarized in table 17 where each figure is the average of 3 determinations.

The uniformity of the results in this experiment was very striking. It seems clear that in alkaline solutions the pH tends to shift towards a neutral zone (pH 7.3 to 7.4); that it shifts more rapidly in bacterial suspensions which are being shaken than in those which are not being shaken; that the pH change is greater or more rapid in the less toxic solutions; and that the shift is greater and more rapid in solutions of NaCl than in solutions of $CaCl_2$ of approximately the same toxicity. These results completely confirm our hypotheses and, it appears to us, explain the apparently anomalous results described above. It is evident that relatively slight differences in the general pH of a solution under such conditions must indicate very great differences in the restricted pH of the zones immediately surrounding the bacterial cells.

It appears then that the toxic effect of 0.145 M $CaCl_2$ is an indirect one and is exerted only in an alkaline solution in which the salt interferes with the regulative action exerted by bacterial cells upon the reaction of such a solution.

In his studies on the phenomenon of agglutination Buchanan (1919) has pointed out that under appropriate conditions and in certain concentrations $CaCl_2$ serves as an agglutinating agent with suspensions of meningococci. This suggests that the apparent reduction in numbers of bacteria in $CaCl_2$ solutions may be attributable to agglutination rather than to true toxicity. Although there may be as many viable cells in an agglutinated suspension as in a non-agglutinated one, the number of colonies appearing on an agar plate will be different in the two cases. Direct microscopic observations of $CaCl_2$ suspensions of *Bact. coli*, however, indicate that with the conditions under which our experiments were conducted agglutination did not ordinarily occur. The rôle of the H-ions present in their influence on agglutinability has not yet, however, been thoroughly studied in this connection.

SUMMARY AND CONCLUSIONS

1. The strain of *Bact. coli* with which we have conducted our experiments maintains itself in distilled water at a favorable pH value without material decrease in numbers for a period of nearly twenty-four hours. Increases are not uncommon during the first few hours. Occasionally however a particular suspension will show a marked decrease due to some cause which we have not yet determined.

2. A reaction of about pH 6.0 is most favorable to the viability of these bacteria in distilled water, the viability decreasing as a solution becomes more acid or more alkaline.

3. A NaCl solution of 0.0145 M strength exerts a distinctly favorable action upon viability. Instead of a slight but definite decrease after twenty-four hours we find that in these highly dilute salt solutions the bacteria maintain themselves in undiminished numbers. The same favorable result is apparent in a $CaCl_2$ solution of 0.00145 M strength.

4. NaCl solutions of 0.725 M strength and over and $CaCl_2$ solutions of 0.435 M strength and over are distinctly toxic at all reactions.

5. Finally a $CaCl_2$ solution of intermediate strength (0.145 M) shows very peculiar and interesting results. At any pH value maintained throughout an experiment by repeated readjustments such a solution is non-toxic. In an unadjusted alkaline solution, however, it displays a definite toxic action which we have demonstrated to be due to the fact that the $CaCl_2$ prevents the bacteria from bringing about a reversion to a more favorable neutral reaction. Our experiments make it clear that in an alkaline solution the bacteria normally alter the medium in the zone immediately adjacent to them in such a way as to decrease its alkalinity to a very marked degree, a process which is inhibited by 0.145 M $CaCl_2$.

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