THE DIFFUSION PRODUCTS OF BACTERIAL CELLS AS INFLUENCED BY THE PRESENCE OF VARIOUS ELECTROLYTES[1]

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INTRODUCTION

The influence of electrolytes upon plant and animal cells has been studied by a great number of observers; and the literature of the subject has been so well reviewed by Falk (1923) that it requires no detailed summary here. In general the influence of the electrolytes has been measured by the growth of cells in favorable media or their survival in unfavorable media or by the rate of chemical change produced through cell activity, in the formation of carbon dioxide from sugar solutions, in the formation of ammonia from pepton or in the process of nitrification. Changes in electrical resistance have also been studied by many observers, with contractility of muscle and viability of eggs and larvae in animal forms. These investigations have, as is well known, justified the broad conclusions that dilute solutions of electrolytes are favorable and stronger solutions unfavorable, to biological processes, that bivalent cations are more powerful in their effects than monovalent cations, that where permeability effects are manifest the univalent cations tend to increase diffusion and the bivalent cations (in similar concentration) tend to decrease it, and that these two types of cations appear to exhibit a more or less definite antagonism.

Since the appearance of Falk's review of the subject the most

[1] Part of a dissertation presented by H. J. S. for the degree of Doctor of Philosophy in Yale University.

important contributions have been along four different lines as follows:

a. Cook (1926) has shown that the production of carbon dioxide by Aspergillus is decreased by H, Cu and Hg ions in the concentrations studied while Ag first increases and then decreases it.

b. The penetration of various substances into the vegetable cell has been studied by direct observation in the case of the giant cells of Nitella and Valonia by Osterhout (1925), Osterhout and Dorcas (1925), Irwin (1926), and M. M. Brooks (1926 a, b). The first three observers claim that the plant cell is penetrated only by undissociated molecules, a conclusion which Mrs. Brooks denies.

c. Various investigators have reported on the effect of electrolytes upon the electrical conductivity of suspensions,—Peters (1908), studying Paramecium; Stiles and Jorgenson (1915) and S. C. Brooks (1917), the tissues of higher plants; Medes and McClendon (1920), Elodea; Gray (1920, 1921), trout eggs; Osterhout (1922), Laminaria; Shearer (1919, 1920), Green and Larson (1922), Johnson and Green (1924), MacDougall and Green (1924), Brooks (1925) and Zoond (1927), bacterial cells. There is considerable question as to the interpretation of much of this work and as Brooks, Green and Larson and others have emphasized increased conductivity may be merely due to exosmosis of electrolytes. Be this as it may it appears that monovalent cations generally cause an increase followed by an irreversible fall. Osterhout and Shearer believe that the dead cell has no resistance but Green and Larson and Zoond report only a very moderate decrease.

d. Finally, a large group of recent workers have studied the effect of electrolytes upon the passage of various anions and cations into and out of erythrocytes. Among the more important of the studies of this type are those of Gürber (1895), Hamburger (1916), De Boer (1917), Van Slyke and Cullen (1917), Fridericia (1920), Doisy and Eaton (1921), Mukai (1921), Wiechmann (1921), Mellanby and Wood (1923), Ashby (1924) and Coulter (1924). In general these observers find that H, Cl and CO_2 migrate very readily back and forth between corpuscles and

serum while Na and K according to most investigators (but not Mellanby and Wood and Ashby), fail to do so.

The present study involves phenomena related in a measure to the last two types of investigation reviewed above. It deals however with specific chemical products of diffusion and not with change in conductivity and with bacterial cells and not erythrocytes. We have sought to determine the effect of electrolytes upon the nature and amount of the products which diffuse outward from the bacterial cell into a surrounding menstruum in which the organisms are either barely surviving or slowly dying out but without active growth processes on the one hand or the action of powerfully toxic agents on the other. It was thought that a study of this type would perhaps throw light upon the question of the influence of electrolytes upon normal metabolism and the behavior of the cell membrane in normal metabolism as distinct from their effect upon enzyme action as manifest in such processes as fermentation or ammonification.

In a previous communication from this laboratory (Shaughnessy and Falk, 1924) it was shown that the cells of *Bact. coli* exert a marked buffering effect when suspended in distilled water, an effect which rises to a maximum in the range favorable to viability (pH 6.0 to 6.9) and which gradually falls off with increasing acidity or alkalinity. Neutral salts (Ca and Na) greatly decrease this buffer action particularly on the alkaline side, although in highly toxic concentration of calcium there is a secondary increase in buffer, probably due to lysis and liberation of buffer substances from the ruptured cell.

This power of the cell to regulate the hydrogen-ion concentration of a surrounding menstruum is one of its most fundamental biological properties. This power is in part exerted by the binding of positive or negative ions by the cell as a whole. It is in part, however, due to changes produced in the adjacent menstruum by the bacterial cells through the discharge of substances into that menstruum. Such a process was demonstrated by Winslow and Falk (1923) who showed that in alkaline solution the bacteria liberate acidic substances which create a zone of lower alkalinity in their immediate vicinity.

In the earlier work a buffering power conducted in this laboratory no distinction could be made between these processes since the influence of the cells upon the menstruum was determined in the presence of the cells which exerted it. The purpose of the present study was to discover the nature of the changes produced in the surrounding medium by analysis of the menstruum after the cells themselves had been removed by centrifuging.

GENERAL TECHNIQUE EMPLOYED IN OUR EXPERIMENTS

Two types of bacteria were used for the present study, one a strain of *Bact. coli* and the other a strain of *B. cereus*. Both have been extensively used in previous investigations made in this laboratory and they have been found to differ from each other in one highly important respect. The *Bact. coli* survives in practically undiminished numbers in distilled water or dilute salt solutions throughout a moderate pH range while the *B. cereus* dies out very rapidly in any such menstruum (not containing organic protective substances) ninety per cent of the cells being non-viable after an hour. We were thus able to compare the reactions of living and dead cells as exemplified by these two types.

The *Bact. coli* was cultivated before each test for twenty-two to twenty-four hours at 37°C. on nutrient agar in Kolle flasks; the *B. cereus* on the other hand was cultivated for fourteen to sixteen hours at 22° to 24°C. (a condition which previous studies had shown would ensure freedom from spores). The organisms were then washed off in distilled water, filtered through cotton to remove any trace of agar, centrifugalized twice at high speed from water and finally resuspended in water. The final suspensions were examined microscopically to make sure of cultural purity and to prove that there were no spores. The concentration of the suspension was then standardized against turbidity standards made from $BaCl_2$ and H_2SO_4 in varying proportions.

The water was freshly distilled from a Barnstead still and gave no test for ammonia with Nessler's reagent. It rapidly reached an equilibrium with the CO_2 of the atmosphere so that its acidity was consistently at pH 5.7 to 5.8. The NaCl was

Baker's Analyzed and the $CaCl_2$ was the same brand, recrystallized from water to rid it of $MgSO_4$. These chemicals were then dried in an electric oven at 105°C. and kept in a desiccator until weighed.

The HCl solutions used for titration and for adjusting the pH of the solutions was standardized by the potentiometric method, using the apparatus described by Shaughnessy and Falk (1924). The NaOH solution used for the titrations was standardized against the HCl solution daily.

All glassware used in these experiments was of Pyrex brand, neutralized and cleaned each time by immersion in sulphuric acid-potassium dichromate solution over night and by subsequent rinsing with running hot tap water with final rinsing in distilled water. It was then thoroughly dried in an electric oven at 105°C. for three to four hours.

EFFECT OF DIFFUSION PRODUCTS UPON THE REACTION OF THE MENSTRUUM

In the conduct of a typical experiment, 1 cc. of a heavy suspension of the organism was added to each of a series of 150 cc. Erlenmeyer flasks, containing exactly 50 cc. of the test solution, which had already been adjusted in bulk to the desired pH with HCl or NaOH,—giving a final concentration of about 500 million cells per cubic centimeter. For each flask of suspension there was also a control flask containing 50 cc. of the test solution of the same pH plus 1 cc. of distilled water. The flasks were very lightly plugged with cotton to prevent the type of autolysis that Jaumain (1922) has observed to occur in tightly plugged tubes and were kept in the incubator at 37°C. for the specified lengths of time. The control flasks were kept under the same conditions as the respective suspensions but were not centrifugalized. The zero time period in the charts and tables corresponds to analyses made from flasks centrifugalized immediately after seeding.

After the organisms were strongly sedimented in the centrifuge, the supernatant fluid was carefully poured off, without disturbing the sediment, into Erlenmeyer flasks of the same size as those containing the control. A measured amount of one of

Clark and Lubs' series of indicators was added to the test and control fluids, respectively, the pH of each recorded and the pH of the test fluid adjusted to that of the control with N/1000 HCl or NaOH as the case might be. In our charts and tables we have given the figures in terms of titratable acid or alkali necessary to cause this change because it was not always possible to measure the pH of the test fluid with the same indicator that was used for the control and because pH values cannot be averaged easily because of their logarithmic nature. In some cases we have made note that the total acid or alkali neutralized and the pH changes fail to harmonize and have indicated our explanation of the phenomenon.

The solutions studied included water and $CaCl_2$ and NaCl solutions (in 0.0145, 0.145 and 1.45 M concentration) adjusted to pH values of 6.0, 7.0 and 8.0. The periods of exposure studied were 0, $\frac{1}{2}$, 1, 2, 4 and 24 hours. Each figure presented in the tables is the average of from two to seven different experiments, the exact number being given in the table in each case.

In addition to the studies made with living *Bact. coli* and with *B. cereus* cells which had died from exposure to an aqueous menstruum we also made some observations on the boiled cells of both species and on *Bact. coli* cells killed by 15 minute exposure to a temperature of 60°C. which we found by plating to be the minimum time in which the organism could be killed at that temperature. The volume of the suspension was of course restored to its original value after heating.

The results of our tests upon *Bact. coli* and *B. cereus* in their normal state and after heating are presented in tables 1 to 8. The data are grouped in the tables according to the organism tested and the reaction of its original menstruum but it will perhaps be more instructive to discuss the results in a different order, taking up first all tests made with water as a menstruum and then considering the effect of electrolytes upon the fundamental reactions involved.

In figure 1 we have presented all the important data which illustrate the effects of the various organisms tested upon the titratable acidic and alkaline substances in water without the

presence of electrolytes. The three sets of curves show reactions in water primarily adjusted to pH values of 8.0, 7.0 and 6.0, respectively. The ordinates above the base lines represent the

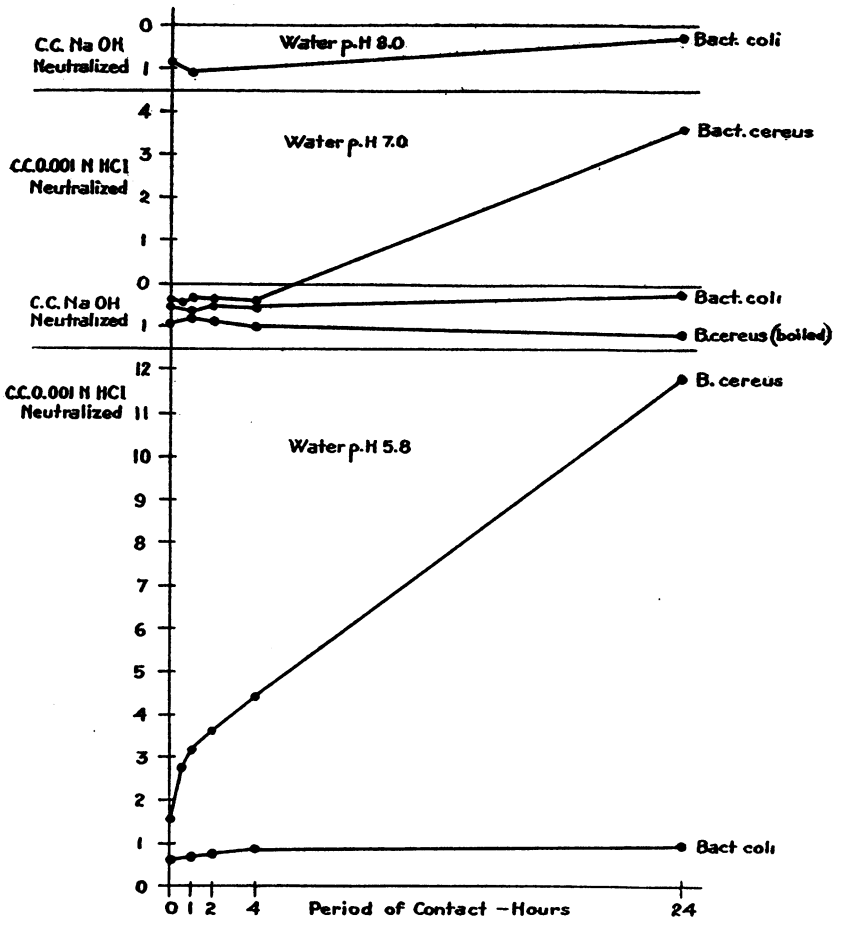

FIG. 1. THE EFFECT OF B. CEREUS AND BACT. COLI UPON THE TITRATABLE ACIDITY OF DISTILLED WATER

amounts of acid necessary to restore the solutions to their original values (or to the value of a control not exposed to bacterial cells). Ordinates below the base line represent amounts of alkali necessary to regain the original pH value.

It will be noted from figure 1 that in alkaline and neutral solution the menstruum in which the bacterial cells have been suspended becomes during the first four hours more acid so that

TABLE 1

Diffusion products of B. cereus in acid menstrua

Alkaline substances formed. In terms of cubic centimeters 0.001 N HCl necessary to neutralize 50 cc.

SOLUTION	PERIOD OF CONTACT						NUMBER OF EXPERIMENTS
	0 hour	½ hour	1 hour	2 hours	4 hours	24 hours	
Distilled water	1.55	2.71	3.21	3.65	4.43	11.78	5
0.0145 M NaCl	0.87	1.77	2.50	3.08	5.18	18.09	5
0.145 M NaCl	0.70	0.90	1.22	1.60	2.12	8.62	5
1.450 M NaCl	0.76	1.19	1.25	1.34	1.31	1.36	5
0.0145 M $CaCl_2$	0.21	0.23	0.42	0.65	1.45	3.77	6
0.145 M $CaCl_2$	0.13	0.01*	0.05*	0.08*	0.10*	0.04	4
1.450 M $CaCl_2$	0.51	0.39	0.34	0.00	0.00	0.13*	3

All solutions started at pH 5.7 to 5.8

* Acidic substance—neutralized with 0.001 N NaOH.

TABLE 2

Diffusion products of B. cereus in neutral menstrua

Acidic substances formed. In terms of cubic centimeters 0.001 N NaOH necessary to neutralize 50 cc.

SOLUTION	PERIOD OF CONTACT						NUMBER OF EXPERIMENTS
	0 hour	½ hour	1 hour	2 hours	2 hours	24 hours	
Distilled water	0.38	0.43	0.37	0.38	0.44	3.55*	6
0.0145 M NaCl	0.95	1.20	1.38	1.76	1.73	4.97*	4
0.145 M NaCl	1.29	2.24	2.08	2.24	2.33	1.14*	6
1.450 M NaCl	0.82	0.80	0.65	0.48	0.66	0.55	5
0.0145 M $CaCl_2$	0.84	1.02	0.96	0.95	0.71	0.28*	3
0.145 M $CaCl_2$	0.72	0.89	1.00	0.96	0.91	0.32	7
1.450 M $CaCl_2$	1.09	1.65	1.52	1.40	2.03	1.86	3

All solutions started at pH 6.8 to 7.0.

* Alkaline substances—neutralized with 0.001 N HCl.

it requires the addition of alkali to bring the solution back to the starting point. In the case of an initially alkaline solution exposed to *Bact. coli* the effect is particularly striking. A wholly

different effect is however manifest after periods longer than 4 hours. At the twenty-four-hour period production of acid is overbalanced by a production of alkali, moderate in the case of

TABLE 3

Diffusion products of Bact. coli in acid menstrua

Alkaline substances formed. In terms of cubic centimeters 0.001 N HCl necessary to neutralize 50 cc.

SOLUTION	PERIOD OF CONTACT					NUMBER OF EXPERIMENTS
	0 hour	1 hour	2 hours	4 hours	24 hours	
Distilled water	0.63	0.70	0.72	0.85	0.93	5
0.0145 M NaCl	0.49	0.50	0.83	0.78	1.69	4
0.145 M NaCl	0.29	0.28	0.48	0.65	1.86	4
1.450 M NaCl	0.09	0.02*	0.00	0.00	0.11*	5
0.0145 M $CaCl_2$	0.29	0.34	0.32	0.33	0.74	5
0.145 M $CaCl_2$	0.40	0.48	0.46	0.45	0.82	3
1.450 M $CaCl_2$	1.15	1.14	1.06	0.54	0.12	3

All solutions started at pH 5.7 to 5.8.

* Acidic substances—neutralized with 0.001 N NaOH.

TABLE 4

Diffusion products of Bact. coli in neutral menstrua

Acidic substances formed. In terms of cubic centimeters 0.001 N NaOH necessary to neutralize 50 cc.

SOLUTION	PERIOD OF CONTACT					NUMBER OF EXPERIMENTS
	0 hour	1 hour	2 hours	4 hours	24 hours	
Distilled water	0.49	0.63	0.46	0.45	0.24	4
0.0145 M NaCl	0.30	0.36	0.49	0.57	0.76	5
0.145 M NaCl	0.53	0.64	0.63	0.55	0.24	3
1.450 M NaCl	0.57	0.58	0.56	0.58	0.52	3
0.0145 M $CaCl_2$	0.47	0.55	0.49	0.56	0.46	3
0.145 M $CaCl_2$	0.55	0.55	0.46	0.42	0.11	3
1.450 M $CaCl_2$	1.12	1.61	1.39	1.45	0.43	4

All solutions started at pH 6.8 to 7.0.

Bact. coli and much more marked in the case of *B. cereus*, the reaction in the latter case actually reaching a pH of 7.4.

It may be noted that these studies in the alkaline medium were rendered very difficult by the continued absorption of

carbon dioxide from the atmosphere. Since our results represent differences between titratable acidity in menstrua containing bacterial diffusion products and in control solutions of the same initial reaction the results are, however, believed to be approximately accurate.

TABLE 5

Diffusion products of Bact. coli in alkaline menstrua

Acidic substances formed. In terms of cubic centimeters 0.001 N NaOH necessary to neutralize 50 cc.

SOLUTION	PERIOD OF CONTACT			NUMBER OF EXPERIMENTS
	0 hour	1 hour	24 hours	
Distilled water	0.89	1.09	0.31	5
0.0145 M NaCl	1.27	0.77	0.15	2
0.145 M NaCl	1.24	1.32	0.47	2
1.450 M NaCl	1.35	1.30	0.33	2
0.0145 M $CaCl_2$	1.36	1.18	0.42	3
0.145 M $CaCl_2$	0.99	0.90	0.25	2
1.450 M $CaCl_2$	3.33	2.86	2.15	2

All solutions started at pH 8.0 to 8.1.

TABLE 6

Diffusion products of heat-killed cells of Bact. coli in neutral menstrua

Acidic substances formed. In terms of cubic centimeters 0.001 N NaOH necessary to neutralize 50 cc.

SOLUTION	PERIOD OF CONTACT				NUMBER OF EXPERIMENTS
	0 hour	1 hour	4 hours	24 hours	
Distilled water	0.52	0.46	0.42	0.26	3
0.0145 M NaCl	0.46	0.42	0.40	0.47	3
1.450 M NaCl	0.52	0.59	0.47	0.58	3
1.450 M $CaCl_2$	1.26	1.43	1.57	0.61	3

All solutions started at pH 6.8 to 7.0.

In the acid solutions the cells of both species cause an immediate and marked production of alkaline substances and this production of alkaline substances continues during the whole period of the experiment, slowly in the case of *Bact. coli* and very rapidly in the case of *B. cereus*.

Heat-killed and even boiled cells of *Bact. coli* (omitted from

the chart; see tables 6 and 7) exert just the same effect as do the living cells but the effect of boiling upon *B. cereus* is to increase the initial production of acidic substances and to check altogether the later reversion to a strongly alkaline reaction.

While preferential absorption of ions may of course play some part in these phenomena, it is clear that we must be dealing

TABLE 7

Diffusion products of cells of Bact. coli, boiled for thirty minutes, in neutral menstrua

Acidic substances formed. In terms of cubic centimeters 0.001 N NaOH necessary to neutralize 50 cc.

SOLUTION	PERIOD OF CONTACT				NUMBER OF EXPERIMENTS
	0 hour	1 hour	4 hours	24 hours	
Distilled water	0.59	0.43	0.72	0.20	3
0.0145 M NaCl	0.57	0.38	0.61	0.52	3
1.450 M NaCl	0.82	0.72	1.00	0.40	3
1.450 M $CaCl_2$	3.54	2.00	3.51	3.33	3

All solutions started at pH 6.8 to 7.0.

TABLE 8

Diffusion products of cells of B. cereus, boiled for thirty minutes, in neutral menstrua

Acidic substances formed. In terms of cubic centimeters 0.001 N NaOH to neutralize 50 cc.

SOLUTION	PERIOD OF CONTACT				NUMBER OF EXPERIMENTS
	0 hour	1 hour	4 hours	24 hours	
Distilled water	0.96	0.76	0.96	1.07	2
0.0145 M NaCl	1.03	1.37	1.54	0.74	2
1.450 M NaCl	1.80	1.32	1.58	1.49	2
1.450 M $CaCl_2$	3.82	3.83	2.75	3.32	2

All solutions started at pH 6.8 to 7.0.

chiefly with the actual elimination into the menstruum by the cell of acidic and basic substances respectively.[2] It appears that the first tendency is toward a liberation of acid substances in a

[2] It has been shown in earlier work from this laboratory (Shaughnessy and Falk, 1924) that the alternative explanation (fixation of basic or acid substances, cannot be in all cases invoked.

neutral or alkaline solution and of basic substances in an acid solution, a process tending to regulate the reaction of the menstruum to the point which we know to be optimum for the life of bacterial cells,—the process described by Winslow and Falk (1923). There seems a close parallelism here between our results and those obtained by Coulter (1924) with red blood cells (an increase in acidity followed by an increase in alkalinity), although his explanation of the phenomenon is different from ours. The second process (alkali production), unlike the first, is checked by boiling.

NATURE OF THE DIFFUSION PRODUCTS WHICH CONTROL THE REACTION OF THE MENSTRUUM

At this point it is worth while to consider the nature of the diffusion products which lead to the changes in reaction which have been discussed. We should naturally expect that carbon dioxide and diffusion of hydrogen alone or in combination with inorganic anions, on the one hand and ammonia on the other hand, would play a part in the changes toward an acid and an alkaline reaction respectively.

a. Hydrogen. The first possible source of acidity would appear to be hydrogen, diffusing out from the cell in combination with basic ions. As an indirect measure of this type of reaction we estimated the chlorides and phosphates contributed to the menstruum by the bacterial cells.

Chlorides were measured by the micro method of Van Slyke (1923) modified so that we used 50 cc. of test fluid and made our determinations with 0.005 N $AgNO_3$ and 0.005 N KCNS in place of the stronger concentrations. The sulfocyanate was checked daily against the silver nitrate and a correction factor made.

The results are presented in tables 9 and 10. It will be noted that in distilled water the diffusion of chlorides is highly variable and never great in amount; but that it is perhaps most marked in an acid menstruum.

Phosphates were measured by the method, credited to Benedict, described in Meyers' (1924) monograph on blood analysis. It consists in clarification of the test fluid with trichloracetic acid,

heating with hydroquinone-bisulfite and molybdic acid reagents and comparison of the cooled solution with a standard phosphate. We modified the procedure so that we could use 25 cc. of the supernatant fluid and control (and still get a good color for comparison) by substituting a phosphate standard made so that 1 cc. = 0.01 mgm. of phosphate. We used 0.0, 0.1, 0.3, 0.5, 0.7, etc., up to 2.5 cc. of this standard diluted to 25 cc. as stand-

TABLE 9

Diffusion products of B. cereus

Chlorides. Expressed as cc. 0.005 N $AgNO_3$ to react with 50 cc.

SOLUTION	PERIOD OF CONTACT				NUMBER OF EXPERIMENTS
	0 hour	1 hour	4 hours	24 hours	
Distilled water (pH 5.7)	0.066	0.049	0.075	0.013	3
Distilled water (pH 7.0)	0.110	0.129	0.139	0.018	3
0.0145 M NaCl (pH 7.0)	0.351	0.333	0.179	0.342	3

TABLE 10

Diffusion products of Bact. coli

Chlorides. Expressed as cubic centimeters 0.005 N $AgNO_3$ to react with 50 cc.

SOLUTION	PERIOD OF CONTACT				NUMBER OF EXPERIMENTS
	0 hour	1 hour	4 hours	24 hours	
Distilled water (pH 5.7)	0.013	0.064	0.052	0.074	3
Distilled water (pH 7.0)	0.007	0.012	0.005	0.002	3
0.0145 M NaCl (pH 7.0)	0.182	0.320	0.263	0.322	3
Cells killed by heating at 60°C. for fifteen minutes					
Distilled water (pH 5.7)	0.039	0.069	0.079	0.074	3

ards in Nessler tubes. Fresh standards were made up daily and, as each group of test fluids at any time period was heated, one new standard was also made to check the equivalent standard in the set already made. By this method we were able to determine 0.001 mgm. of phosphate readily.

The results for distilled water (see tables 11, 12 and 13) show as in the case of chlorides an almost insignificant increase in basic ions. It would seem from these data that in the absence

of added electrolytes the changes in the menstruum effected by the bacterial cell in the direction of acidity are not in large

TABLE 11

Diffusion products of B. cereus

Phosphates. Milligrams per 25 cc.

SOLUTION	PERIOD OF CONTACT				NUMBER OF EXPERIMENTS
	0 hour	1 hour	4 hours	24 hours	
Distilled water (pH 5.7)	0.001	0.002	0.004	0.007	3
Distilled water (pH 7.0)	0.001	0.003	0.006	0.010	3
0.0145 M NaCl (pH 7.0)	0.002	0.003	0.016	0.019	3
1.450 M NaCl (pH 7.0)	0.001	0.004	0.009	0.011	3

TABLE 12

Diffusion products of Bact. coli

Phosphates. Milligrams per 25 cc.

SOLUTION	PERIOD OF CONTACT				NUMBER OF EXPERIMENTS
	0 hour	1 hour	4 hours	24 hours	
Distilled water (pH 5.7)	0.001	0.001	0.001	0.003	3
Distilled water (pH 7.0)	0.001	0.002	0.002	0.002	6
0.0145 M NaCl (pH 7.0)	0.003	0.002	0.003	0.008	4
1.450 M NaCl (pH 7.0)	0.001	0.001	0.002	0.005	3
0.0145 M $CaCl_2$ (pH 7.0)	0.000	0.002	0.002	0.001	3

TABLE 13

Diffusion products of cells of Bact. coli, boiled for thirty minutes, in neutral menstrua

Phosphates. Milligrams per 25 cc.

SOLUTION	PERIOD OF CONTACT				NUMBER OF EXPERIMENTS
	0 hour	1 hour	4 hours	24 hours	
Distilled water	0.001	0.002	0.003	0.003	2
0.0145 M NaCl	0.002	0.003	0.004	0.006	3
1.450 M NaCl	0.001	0.002	0.002	0.000	3

All solutions started at pH 6.8 to 7.0.

measure due to diffusion of hydrogen in combination with basic ions. Chlorides and phosphates were selected for this study because they are the only anions present in appreciable amount

which would be likely to carry hydrogen out from the cell. Guillemin and Larson (1922) have shown that the SO_4 ion is present in bacterial cells in negligible amount.

It appears from these results that the processes of diffusion and the regulative action upon the menstruum must be quite different in bacteria and in red blood cells,—as one might naturally expect would be the case. Gürber (1895), Hamburger (1916), DeBoer (1917), Van Slyke and Cullen (1917), Fridericia (1920), Doisy and Eaton (1921), Wiechmann (1921), Mukai (1921) and Mellanby and Wood (1923) and Coulter (1924) all maintain that chlorin passes in some form rather readily into and out of the erythrocyte. Osterhout (1922) and Irwin (1923a) on the other hand find that Nitella cells are not easily penetrated by chlorides which certainly seems to be the case with the bacteria.

b. Carbon dioxide. The study of carbon dioxide as a factor in regulating the reaction of a solution is surrounded by very great difficulties on account of the constant adjustment which takes place between the concentration of this substance in solution and in the adjacent atmosphere. Furthermore, the course of this process is also materially influenced by the presence of electrolytes in the solution itself, a fact which probably accounts for some of the phenomena observed by Winslow and Falk. The three solutions which best illustrate the effect of buffer upon viability are water, 0.145 M NaCl and 0.145 M $CaCl_2$. We find that if these three solutions be adjusted to pH 8.0 and allowed to stand, without bacterial cells present, for one hour their reactions will change respectively to 7.3, 7.3 and 7.8 under the direct influence of atmospheric CO_2 alone.

For these reasons we decided that it would be useless to attempt any direct quantitative measurements of carbon dioxide and resorted instead to the indirect process of removing the carbon dioxide present by blowing carbon-dioxide free air through the medium and then determining the pH at equilibrium in the carbon-dioxide-free solution, an equilibrium presumably determined by mineral acids. This process would of course remove other acids than carbon dioxide if they were of a volatile or readily oxidizable nature. The extensive oxidation of any acids

present in a period as short as one hour is exceedingly improbable. The introduction of volatile bases was prevented by drawing the incoming air through concentrated sulphuric acid. Our complete equipment consisted of a train of four wash bottles containing respectively soda lime, concentrated NaOH, concentrated H_2SO_4 and distilled water, through which we bubbled compressed air before it was admitted to the test solution.

Another factor came to the surface as soon as we attempted to measure the change in acidity of the solutions. We found that our test and control solutions not only returned to the control pH (neutrality) but became alkaline. The reason for this was not long in disclosing itself. Our distilled water before adjustment was at pH 5.8 due to equilibrium with the atmospheric CO_2 (a fact noted by Loeb, 1922, as well as by many others) and, when we brought it to neutrality, we had created a solution containing $NaHCO_3$ and perhaps Na_2CO_3. At any rate, when we blew off CO_2, the NaOH was again liberated and made the solution alkaline. It was possible, however, to compare the solutions in regard to the final pH reached.

By this method we found that water, 0.0145 M NaCl and 1.450 M NaCl which had been in contact with either *Bact. coli* or *B. cereus* came to the same pH equilibrium point (measured colorimetrically) that the controls did. 1.450 M $CaCl_2$ alone became less alkaline by 0.1 to 0.2 pH units than its control. We shall return to this last phenomenon later.

The net result of this part of our study was to indicate that the acidity produced in a surrounding menstruum by the bacterial cell is only in small measure due to the diffusion of hydrogen ions, alone or in combination with mineral anions, but is chiefly due to volatile acidic compounds of which carbon dioxide is undoubtedly the chief representative.

c. Ammonia. In considering the source of alkaline changes in the menstruum studied the first logical point of attack would seem to be ammonia. Coulter (1924) believed that such a change in his experiments was caused by ammonia production while Brooks (1923b) believed it to be due to diffusion of basic ions; neither had succeeded in demonstrating either fact.

It was apparent that it was useless as well as impossible in the time available to measure the production of ammonia in all the solutions studied. For that reason we selected for this and for all

TABLE 14

Diffusion products of B. cereus

Ammonia nitrogen. Milligrams per 50 cc.

SOLUTION	PERIOD OF CONTACT				NUMBER OF EXPERIMENTS
	0 hour	1 hour	4 hours	24 hours	
Distilled water (pH 5.7)	0.006	0.009	0.011	0.070	3
Distilled water (pH 7.0)	0.008	0.009	0.018	0.090	3
0.0145 M NaCl (pH 7.0)	0.009	0.014	0.035	0.150	3
1.450 M NaCl (pH 7.0)	0.000	0.000	0.000	0.000	3

TABLE 15

Diffusion products of Bact. coli

Ammonia nitrogen. Milligrams per 50 cc.

SOLUTION	PERIOD OF CONTACT				NUMBER OF EXPERIMENTS
	0 hour	1 hour	4 hours	24 hours	
Distilled water (pH 5.7)	0.004	0.006	0.009	0.019	3
Distilled water (pH 7.0)	0.005	0.006	0.008	0.020	3
0.0145 M NaCl (pH 7.0)	0.007	0.008	0.011	0.034	3
1.450 M NaCl (pH 7.0)	0.000	0.000	0.000	0.000	3

TABLE 16

Diffusion products of cells of Bact. coli, boiled for thirty minutes, in neutral menstrua

Ammonia nitrogen. Milligrams per 50 cc.

SOLUTION	PERIOD OF CONTACT				NUMBER OF EXPERIMENTS
	0 hour	1 hour	4 hours	24 hours	
Distilled water	0.002	0.002	0.002	0.002	3
0.0145 M NaCl	0.003	0.003	0.004	0.012	3
1.450 M NaCl	0.000	0.000	0.000	0.000	2

All solutions started at pH 6.8 to 7.0.

of our later work the two salts which showed the greatest changes, i.e., 0.0145 M NaCl and 1.450 M NaCl, at neutrality and water at acid and neutral reaction. It was found that the $CaCl_2$ solutions

precipitated with the Nessler reagent so it was necessary to use 1.450 M NaCl instead of the same strength of $CaCl_2$.

The measurement of ammonia production was made by the method of direct Nesslerization given in Standard Methods of Water Analysis of the American Public Health Association, 1923, page 16, except that preliminary clarification was found to be unnecessary. The results are expressed in terms of milligrams $\times 10^{-3}$ of ammonia nitrogen for each 50 cc. of solution.

The results of these determinations are presented in tables 14 to 16. They indicate a very considerable production of ammonia when the living cells of either *Bact. coli* or *B. cereus* had been present. The smallest quantity recorded (0.004 mgm.) is equal to approximately N/200,000 solution of NH_4OH. The boiled cells of *Bact. coli* show a very marked inhibition of this ammonia formation.

It is also highly significant to note that the output of ammonia is about the same in acid and in neutral solution. The very different effect upon net reaction in these solutions must, then, be due to a balance between the production of ammonia and of carbon dioxide.

So far as *B. cereus* is concerned, it is clear that the production of ammonia must be due to the action of autolytic enzymes rather than to normal metabolism since the cells are killed by the conditions of the experiments. The important part played by NH_3 and CO_2 in the phenomena studied is in harmony with the findings of almost all those who have observed similar phenomena in either plant or animal cells. Rous (1925) has shown that, as one would expect, the tissue cells of mammals show the greatest readiness in the giving up and taking in of CO_2. Red blood cells of course exhibit the same phenomena in high degree. Brooks (1923a), working with Valonia cells demonstrated the ready passage of NH_3 and CO_2. Harvey (1911, 1913, 1914) showed that ammonia readily diffused into the cells of Elodea, Spirogyra and Paramoecium and McCutcheon and Lucke (1924) and Irwin (1925) have confirmed the penetration of NH_3 into starfish eggs, Gonionemus and Nitella cells.

EFFECT OF HEAT KILLING UPON DIFFUSION FROM THE BACTERIAL CELL

While the cells of *B. cereus* (which die under the conditions of these experiments) are much more permeable than those of *Bact. coli* which survive, the diffusion of acidic substances from the bacterial cell appears to go on at about the same rate from the heat killed and boiled cells as from the living cells of *Bact. coli* (compare tables 6 and 7 with table 4 and table 8 with table 2). This seems at first somewhat surprising but Osterhout (1922) records results which demonstrate that production of CO_2 may continue or even increase after death. This process might be due to decarboxylization but seems to be very rapid for such a mechanism. The diffusion of chlorides is also uninfluenced by heating (see table 10).

The production of ammonia is on the contrary almost abolished by heating as shown by a comparison of table 16 with table 15 and the same phenomenon is apparent in the abolition of the reversion after twenty-four hours to an alkaline condition, for *B. cereus* in the last column of table 8 as compared with the last column of table 2. Evidently the liberation of ammonia is intimately related to a process which is absent in the heat killed cell but not in the cell of *B. cereus* which has died from exposure to an aqueous menstruum. Apparently the enzymes which cause liberation of ammonia operate after the natural death of *B. cereus* cells in aqueous menstrua but are destroyed by heat.

It will be recalled that Shearer (1919, 1920) found the electrical resistance of meningococci killed by exposure to unfavorable menstrua was abolished by death while Green and Larson (1922) and Zoond (1927) found only a slight decrease in heat killed cells of other bacterial species. Winslow and Willcomb (1905) found that, while heat-killed bacterial cells remain stainable, cells which die normally in an unfavorable menstruum lose their staining properties almost at once. It is clear that "death" may be accompanied by widely different types of physical change in the cell membrane under different conditions. It seems probable that when bacteria die in a slightly unfavorable menstruum

their permeability is likely to increase while heat killing produces no such effect. On the other hand, ammonia production shows a precisely opposite effect, proceeding normally in the cells of *B. cereus* which have died normally in an unfavorable menstruum but ceasing in heat-killed cells.

THE INFLUENCE OF ELECTROLYTES UPON THE DIFFUSION PRODUCTS OF THE BACTERIAL CELL

The data so far discussed indicate that the bacterial cell in aqueous suspension first regulates the reaction of the surrounding menstruum toward an optimum point by the diffusion into the menstruum of ammonia and of carbon dioxide and other volatile acids, balanced according to the original reaction of the solution. Thereafter, there occurs a liberation of an excess of ammonia, causing a progressive swing toward alkalinity which is very marked in the case of *B. cereus* (an organism which dies out rapidly during the course of the experiment).

Our next problem concerned the influence of other electrolytes (Na and Ca) upon the course of these fundamental reactions.

Taking first the primary production of acidic substances in initially neutral or alkaline solutions we note that in the case of *Bact. coli* (tables 4, 5, 6 and 7) no important influence is exerted by any of the salts studied except the strong $CaCl_2$ (1.450 M). The latter solution causes a sharp increase in titratable acidity which, however, becomes less marked with the passage of time. We are inclined to attribute this increase in titratable acidity to the liberation of protein buffers by lysis of the cells, particularly as the pH does not show any corresponding increase. This conclusion has been confirmed by tests for protein by biuret and Millon tests which show protein to be present in the strong calcium solutions but not in any of the others. The later decrease in titratable acidity in these calcium solutions may perhaps be attributed to the accumulation of non-reactive surface films on the protein micellae due to contact with the solution or to later absorption of the oppositely charged ions.

Turning now to the production of alkaline substances we note that both *B. cereus* (table 1) and *Bact. coli* (table 3) in acid solu-

tions show the same general phenomena. With *B. cereus* (table 1 and fig. 2) the dilute NaCl (0.0145 M) increases the amount of alkaline substances, diffused while all the other salts decrease it

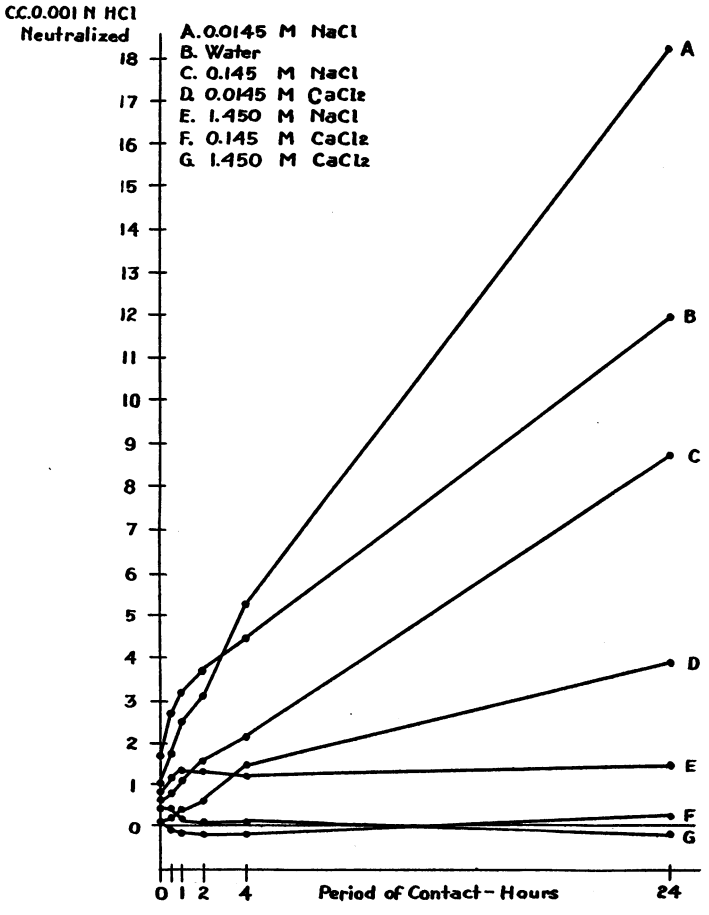

FIG. 2. THE EFFECT OF B. CEREUS UPON THE TITRATABLE ACIDITY OF ACID SOLUTIONS

in approximate proportion to their concentration, the order of effectiveness being 0.145 M Na, 0.0145 M Ca, 1.45 M Na, 0.145 M Ca and 1.45 Ca.

With *Bact. coli* (table 3 and fig. 3) the same general phenomena

appear, although both 0.145 M Na and 0.0145 Na increase diffused alkali and 0.145 Ca shows a slightly less decrease than does 0.0145 Ca. With both organisms the strong Ca solution differs from all others in showing an initial excess of alkalinity followed by a decrease, probably due to the phenomenon described above,—lysis of cells with liberation of buffer, followed by formation of surface films. The effect is more marked with *Bact. coli* than with *B. cereus* possibly because the cell wall of the latter organism is so much more permeable that it is less readily ruptured.

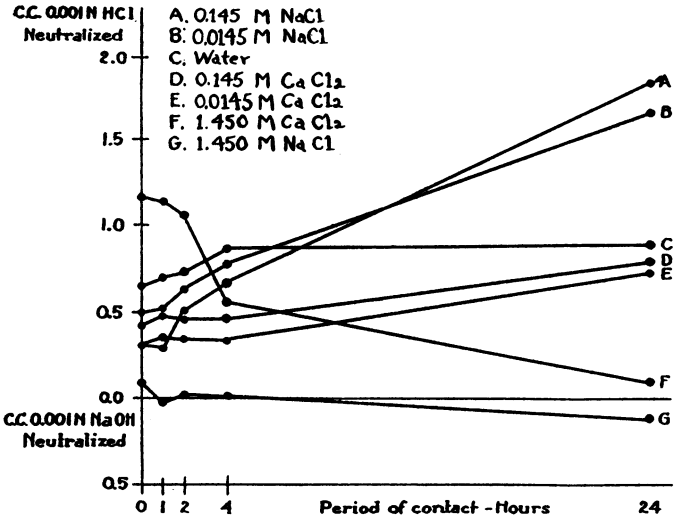

FIG. 3. THE EFFECT OF BACT. COLI UPON THE TITRATABLE ACIDITY OF ACID SOLUTIONS

The cells of *B. cereus* in neutral solution (table 2 and figure 4) show a somewhat more complex relationship but one that is easily explained on relatively simple assumptions. The aqueous solution and the solution of 0.0145 Ca, 0.145 Ca and 1.450 M Na show a progressive production of alkaline substances; most marked in the water and decreasing with the stronger salts. The 1.450 M Ca solution shows the usual initial increase in titratable acidity due to protein buffers. The weak Na solutions on the other hand exhibit a reverse curve corresponding to an

initial increase in liberation of acidic substances during the first four hours followed by a greatly increased liberation of alkali during the subsequent period. We interpret this as due to the fact that dilute sodium salts increase the permeability of the cell wall,—both to the acidic substances first set free and to

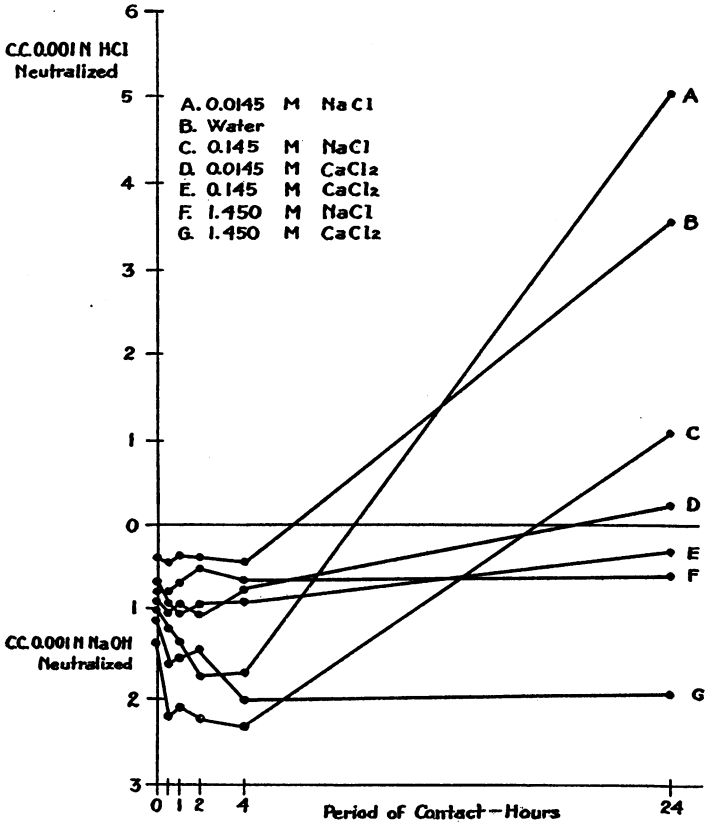

FIG. 4. THE EFFECT OF B. CEREUS UPON THE TITRATABLE ACIDITY OF NEUTRAL SOLUTIONS

the ammonia which is diffused during the later stages of the process.

The influence of electrolytes upon the production and diffusion of alkaline substances is further illustrated by the data for

ammonia, presented in tables 14, 15 and 16 and in figure 5. The weak Na solution (0.0145 M) increases the diffusion of ammonia and the strong solution (1.450 M) practically abolishes it. The latter result may be due to diminished permeability of the cell

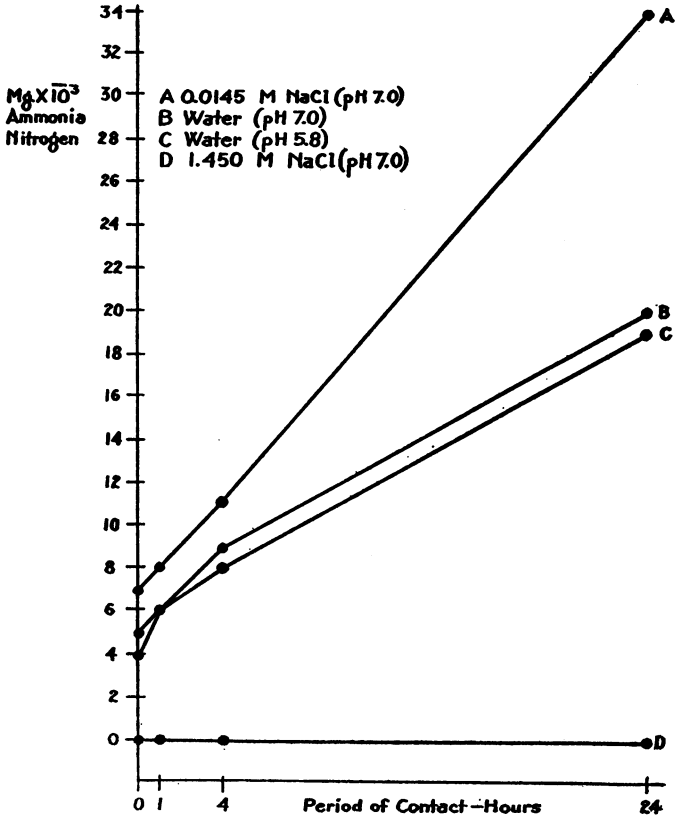

FIG. 5. THE EFFECT OF BACT. COLI UPON THE AMMONIA CONTENT OF MENSTRUA

wall or to diminished production of ammonia within the cell due to inhibition of enzyme action. Lipman (1909) has reported that production of ammonia from peptone is markedly decreased by strong NaCl.

So far as chlorides are concerned tables 9 and 10 again illustrate

the fact that a weak Na solution (0.0145 M) greatly increases the permeability of the cell wall of both *Bact. coli* and *B. cereus* as evidenced by the marked increase of chlorides present in the menstruum. It was not possible to test the effect of high concentrations of salts in the menstruum because the large initial addition of chlorides which such an experiment involves would mask any changes due to the bacterial cells.

It is of special interest to note that cells killed at 60°C. for fifteen minutes (table 10) did not show any greater diffusion of chlorides than did the normal cells, a fact opposed to the usual view that rapid exosmosis occurs upon cell death. The data for phosphates presented in tables 11, 12 and 13 show again that dilute NaCl (0.0145 M) increases diffusion.

The heat-killing or boiling of the cells produces no effect in the salt solutions different from that exerted in water,—with one single exception. In general, ammonia production is checked and carbon dioxide production unaffected in salt solutions exposed to heat killed cells as compared with those exposed to normal cells. The strong $CaCl_2$ (1.450 M) however in the case of boiled cells (table 7),—but not on the case of cells killed at 60° (table 6), shows a marked excess of acid production in comparison with living cells (table 4). We are inclined to attribute this to hydrolysis of the proteins liberated by lysis under the action of the strong calcium solution.

The general results obtained in regard to the influence of electrolytes upon the processes studied are in full accord with the findings of earlier observers. Thus, the fact that dilute solutions of electrolytes increase, while stronger solutions hinder, diffusion was demonstrated by Endler (1912) for green algae, while S. C. Brooks (1916, 1917) with dandelion tissue, Shearer (1919, 1920) with bacteria, Osterhout (1922) with Laminaria and Winslow and Falk (1923) with bacteria all report that Na tends to increase and Ca to decrease permeability. Shearer and Osterhout record a subsequent increase in permeability due to prolonged effect of the bivalent ion which may, as we suggest, be explained by actual rupture of the cell wall.

SUMMARY AND CONCLUSIONS

1. The earlier work of Shaughnessy and Falk (1924) has shown that cells of *Bact. coli* in the zone of physiological interest possess appreciable capacity to resist changes in reaction in such a manner as to avoid injury to the cell. We now find that, in addition to whatever direct absorption of H or OH ions may take place, the cells exert a distinct influence upon the reaction of the menstruum which may be measured by direct chemical tests in this menstruum after the removal of the cells. This type of action involves the liberation of acidic substances in alkaline or neutral medium and of alkaline substances in a more acid medium, the process being so balanced that the ultimate acidity approximates the zone of hydrogen ion concentration (pH 6.2 to 6.4) most favorable to viability (early results for distilled water in tables 1, 2, 3 and 4). This may be interpreted as an adaptive reaction, favorable to the life of the cell.

2. On more prolonged exposure to a somewhat unfavorable aqueous menstruum the production of ammonia overbalances that of acidic substances and in the case of the cells of *B. cereus*, which die under the conditions of the experiment, the excess of alkali liberated may be very large. This reaction is undoubtedly characteristic of injury and interference with the normal life of the cell.

3. The cells of both *Bact. coli* which survive and of *B. cereus* which die out in this menstruum appear to be relatively impermeable to chlorine and phosphate ions and also to calcium ions. The results of two series of studies on calcium have not been cited above in detail but showed insignificant amounts of this ion in menstrua exposed either to *Bact. coli* or *B. cereus*. On the other hand the cell wall allows free passage of carbon dioxide and ammonia and it is to these substances that the effects upon the menstruum are chiefly to be attributed,—the primary adaptive regulation being due to a balanced production of CO_2 and NH_3 the later alkalinity to an excess of NH_3.

4. In general the cell wall of *B. cereus* is obviously much more permeable than that of *Bact. coli*, a phenomenon probably related

to the fact that this organism promptly succumbs in aqueous suspension while *Bact. coli* survives in almost undiminished numbers. It seems more appropriate to assume that this organism dies because it is highly permeable than that it becomes permeable because it dies, but in any case the type of cell death leaves NH_3 production unimpaired. On the other hand death of cells due to heating at 60° for fifteen minutes or boiling for thirty minutes does not increase the diffusion of the electrolytes studied; nor does it interfere with the liberation of carbon dioxide: while it amost wholly inhibits the production of ammonia.

5. *a.* Dilute solutions of sodium chloride (0.0145 M and generally 0.145 M) tend to increase the permeability of the cell wall and to promote the diffusion of all the products studied,—ammonia and other alkaline substances (tables 1, 2, 3, 14, 15 and 16), carbon dioxide (tables 2, 4, 6 and 7) chlorides (tables 9 and 10) and phosphates (tables 11, 12 and 13) whether in the presence of normal or heated cells. It is interesting to note that this increase in permeability occurs in a salt solution which we know to be highly favorable to the viability of *Bact. coli*, indicating that increased permeability may be favorable rather than unfavorable to cell life. (In the case of CO_2 it must always be remembered that increased liberation of the substance may be, in part or in whole, due to increased production within the cell rather than to increased permeability.)

b. A strong solution of NaCl (1.450 M) and solutions of $CaCl_2$ of moderate strength (0.0145 M and 0.145 M) on the other hand decrease the liberation of ammonia and other alkaline substances (tables 1, 2, 3, 14, 15 and 16); but increase the liberation of acidic substances (see first part of table 2 and tables 4, 5, 6, 7 and 8). It may well be that the latter phenomenon is really only the result of the former or, in other words that these salts merely check ammonia formation and leave carbon dioxide production and diffusion unaffected. Our tests with phosphates suggest that 1.450 M NaCl may slightly increase permeability (tables 11 and 12).

c. Finally the strong calcium solution (1.450 M) shows with *Bact. coli* a sharp initial rise in titratable alkalinity (table 3)

and in titratable acidity (tables 4, 5, 6, 7, and 8) followed by a fall, which we interpret as due to a decrease in permeability leading to lysis and liberation of proteins followed by an accumulation of non-reactive films on the protein micellae or to absorption of the oppositely charged ions. An alternative explanation would be that strong $CaCl_2$ causes a type of membrane coagulation which opens the cell wall to the free passage of substances to which it is impermeable. In any case it is highly significant that the highly permeable membrane of *B. cereus* gives no such reactions,—the strong calcium solution (1.450 M) showing here only the increase of acidic substances and the decrease in ammonia manifest in weaker solution, though in more marked degree (tables 1 and 2).

6. Finally, we have been impressed throughout this and earlier work with the fact that the current assumption of an inherent antagonism between monovalent and bivalent ions may perhaps be an unnecessary one, at least so far as bacterial cells are concerned.

Very dilute sodium salts behave in one way and very strong calcium salts in another; and at a given molar concentration different effects and sometimes opposite effects may be manifest; but intermediate strengths of both salts may exert an essentially similar influence. May it not be possible to explain the observed phenomena on the assumption that dilute solutions of either Ca or Na tend to increase permeability and that strong solutions of either salt tend to increase it, the concentrations of NaCl to produce a given effect being of course much higher than the corresponding concentrations of $CaCl_2$? Such an assumption would be in accord with the work of Endler (1912) on the influence of salts upon the absorption and diffusion of dyes and with the work of many other observers.

In order to test this hypothesis we have employed the empirical test devised by Mines (1912) which indicates, as he believes, whether a suspended substance is in an emulsoid or in a suspensoid state. Heavy suspensions of *Bact. coli* were prepared in water and in various salt solutions and stored for one-half hour at 37°C. Varying amounts (best 3 drops) of cobalt-hexamin-

chloride (luteo-cobalt chloride) and of 0.1 M aluminum chloride were then added to the suspensions and they were again stored for twenty hours, at which time the amount of agglutination was determined macroscopically. According to Mines (1912) and Oliver and Barnard (1925) absence of precipitation in the cobalt solution and strong precipitation in the aluminum solution indicates the presence of emulsoids while strong agglutination in both solutions indicates the presence of suspensoids. Our tests indicated that in the presence of 0.0145 M, and 0.000145 M $CaCl_2$ the bacterial substances behaved as emulsoids, while in water and strong salt solutions (1.450 M NaCl and 0.00145–1.450 M $CaCl_2$) they reacted as suspensoids.

If such an explanation should prove to be justified a substantial simplification of our conception of salt action should result.

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