SALT EFFECTS IN BACTERIAL GROWTH[1]

I. PRELIMINARY PAPER

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The Hofmeister series shows the effects of ions of neutral salts upon the coagulation of colloids and upon the swelling and other physical properties of proteins. Our knowledge of these ion effects in solution has been greatly extended by Freundlich and his students. They noted that the ions could be arranged in a definite order with respect to their effects upon compressibility, surface tension, solubility, viscosity, absorption, ratio of reaction, etc. Freundlich seems to favor the hydration theory of salts as an explanation of this neutral salt action, and since the properties affected are so closely related and bound up with one another, and the ions so consistent in their order of effect, he calls these effects "lyotropic" effects. The lyotropic explanation does not lay claim to being a full explanation of neutral salt action, but it does lay claim to correctness in that it systematically treats complicated phenomena.

In most cases the influence of the anion far outweighs that of the cation and the order of anion effects usually reads as follows, $F > SO_4 > PO_4 > Cl > NO_3 > Br > I > CNS$; while the order of arrangement for the cation is usually $Ca > Sr > Mg > Cs > Rb > K > Na > Li$. The same sequence is obtained in the widely differing changes mentioned above and does not seem to follow any recognizable order with respect to valency, atomic weight, etc. Certain reagents may promote or hinder the salt effects as compared with those in pure solution. In some cases the

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order of effects may be reversed when changes take place in acid or in alkaline solutions, but the sequence usually remains the same.

The lyotropic effects of salts upon compressibility, surface tension, solubility, etc., of organic and inorganic substances in solution is not great in most cases. It is in the field of colloid chemistry that these effects attain a magnitude of great significance. A review of the literature covering this field is out of the question here, but a few citations from the biochemical field will serve to show the reasons for extending the work to the field of bacteriology.

In view of the recent and extensive investigations of Loeb (1918–1921) upon the physical and chemical properties of proteins it will probably be necessary to modify certain conceptions now held concerning the relative magnitude of various ionic effects in protein solutions. As to whether the Hofmeister series of ions will be shown to be entirely a delusion, as is believed by Loeb, we do not care to express an opinion, and it is not the purpose of the present paper to take sides on that controversial question.

Whatever may be the status of ion effects in protein chemistry, specific ionic effects in biological phenomena have been well established by the work of Loeb and many others. It is our purpose to study systematically salt effects, especially the qualitative and quantitative relationships of radicals (anions and cations), as related to bacterial growth, and to correlate these findings with other effects which have been noted in pure chemistry as well as in biology.

Closely related to the phenomena of hydration and coagulation is that of permeability and diffusion. On the basis of the view of Bechhold and Ziegler (1919) that membranes do not act like sieves, but as though they were a network of arranged ions, it is easy to conceive of enormous salt effects upon permeability, both by influence upon ions which are to diffuse and by effects upon ions forming the membrane network.

Bacteria perhaps represent matter in a state as near the state of colloids and also as near the state of living protoplasm as any organism does. The effects of salts should therefore not only be very marked but might reach magnitudes that ought to be taken into account in the culture of bacteria.

Brooks (1919) found that NaCl and KCl in concentrations of 0.15 to 0.20M increased the rate of respiration of *B. subtilis*, while in higher concentrations they decreased the rate. $CaCl_2$ increased the rate in a concentration of 0.05M and decreased the rate in higher concentrations. Dealing with the respiration of *Aspergillus niger*, Gustafson (1919) likewise found a stimulation by NaCl in concentrations of 0.25 to 0.5M and by 0.5M $CaCl_2$.

The work of Winslow and Falk (1919) shows that NaCl and $CaCl_2$ both increase the mortality of *Bact. coli* in water. In the case of NaCl 5 isotonic was distinctly lethal, while in the case of $CaCl_2$ 0.1 isotonic was injurious.

Greaves (1916) found the toxicity of anions as measured by ammonification in soils to be in the following order: $Cl > NO_3 > SO_4 > CO_3$. He also noted that the toxicity of some salts increases more rapidly with increased concentration than does that of others. This action he ascribes to the physiological factor of the organism rather than to the osmotic pressure or salt action of the solution.

The influence of alkaline salts upon phagocytosis was found by Radsma (1920) to depend mainly upon the anions but also somewhat upon the cations. Radsma explains the effect as surface action and considers it an indication of colloidal chemical structure of protein substances at the surface.

Mathews (1906) pointed out that the action of salts upon the protoplasmic system is due chiefly to the ions of the salts and he considers the physiological action dependent upon the available potential energy.

Whether or not the salt action upon bacteria is due to the available potential energy of the ions we shall not attempt to decide. We merely wish to point out in this paper that salts do affect bacterial growth much in the same manner as they affect chemical reactions, coagulation, permeability, etc., that this effect is modified by the hydrogen ion concentration of the medium, and that such effects are probably great enough to be given consideration in bacterial culture.

EXPERIMENTAL

The organism used was *Bact. coli*, and the basic medium chosen was a 1 per cent pepton solution to which was added the crystalline salts in amounts necessary to give the desired concentrations. The media were autoclaved and filtered in case of the formation of a slight precipitate. At this point the pH was adjusted if necessary with HCl or NaOH, and the media tubed and sterilized. These tubes containing 10 cc. were in each case inoculated with a loopful of a young culture and incubated at 37°C.

There was some question as what should constitute a measure of bacterial growth and what factor would be constant enough for comparative purposes. The reduction of methylene blue was first tried. In this case a layer of paraffin oil was used to prevent oxidation by the air. Although it worked quite satisfactorily, it was found that reoxidation occurred in the cases where bacterial action was slow, and thus, instead of giving a sharp end point, really increased the time for reduction. The rapidly growing cultures gave a sharp end point. It was noticed, however, that a slight turbidity was apparent in most cases before reduction could be detected. It was decided, therefore, to use the first sign of turbidity as an indication of the rapidity of bacterial growth.

A few trial experiments indicated that the two methods of detecting growth checked very well, except for the fact that turbidity was first detected and proved a sharper measure than reduction. The first sign of turbidity when the tubes were held against a strong artificial light was therefore used to measure rapidity of growth. This method was further verified by growing the same organism in a medium of 1 per cent pepton containing 1 per cent lactose and adjusted to a pH of 7.0. The production of acidity paralleled the results obtained by reduction and visible turbidity.

Inasmuch as the anionic effects seem to be predominant in chemical reactions it was decided to try the effects of salts having a common cation (sodium). The salt concentration chosen to be used was 0.20 molar, which was low enough to give the ionic effects and not too pronounced osmotic effects of the salts. To eliminate as nearly as possible H-ion effects the pH was adjusted to 7.0, colorimetrically, before final sterilization.

TABLE 1

Showing the effect of various sodium salts upon the rate of growth of Bact. coli

MEDIUM	pH	TIME REQUIRED TO SHOW TURBIDITY
		hours
1 per cent pepton	7.2	4½
1 per cent pepton 0.20 M NaCl	7.3	3¼
1 per cent pepton 0.20 M NaI	7.3	3½
1 per cent pepton 0.20 M $NaNO_3$	7.3	3½
1 per cent pepton 0.20 M Na_2SO_4	7.0	4
1 per cent pepton* 0.20 M Na H PO_4	7.3	4¼
1 per cent pepton† 0.20 M Na lactate	7.0	4½
1 per cent pepton 0.20 M Na oxalate	7.0	9½
1 per cent pepton 0.20 M Na acetate	7.0	10½
1 per cent pepton 0.20 M Na citrate	7.3	10½
1 per cent pepton 0.20 M Na fluoride	7.4	48

* Mono-and di-sodium phosphate were mixed in proper proportions to give a pH of approximately 7.0.

† The sodium lactate used was prepared by adding NaOH to lactic acid until a pH of 7.0 was reached.

Table 1 shows the effects of various sodium salts upon the growth of *Bact. coli*. The table indicates that the Cl, I, NO_3, SO_4, PO_4, and lactate ions accelerate growth of *Bact. coli*, while the other ions tried inhibit to a greater or less extent. Using the Cl, I, SO_4, and lactate ions in the same concentrations, the series was repeated with the following results:

Medium	*Time hours*
1 per cent pepton	4½
1 per cent pepton 0.20 M NaCl	3½
1 per cent pepton 0.20 M NaI	3½
1 per cent pepton 0.20 M Na lactate	3½
1 per cent pepton 0.20 M Na_2SO_4	4½

Table 1 indicates that there is a marked effect of salts upon the growth of *Bact. coli*, and it would seem that it is largely due to the anion.

To find out to what extent the cation affects such growth the effect of the following salts were tried: KCl, NaCl, NH_4Cl, $MgCl_2$, $CaCl_2$, and $FeCl_3$. Table 2 gives the effects of 0.20 molar concentrations of these salts upon growth. Table 2 seems to indicate that there is little difference between the effects of the Na, K, and NH_4 ions. Since in the case of $MgCl_2$ we have twice the concentration of Cl ions which we have in the former, a true comparison cannot be made if the anionic effects predominate. To make our experiments comparable we compared growth in a 0.20 molar NaCl pepton medium with growth in 0.10 molar $MgCl_2$ pepton medium. The effects of 0.40 molar NaCl and 0.20 molar $MgCl_2$ were also tried.

TABLE 2

Showing the effect of various cations upon the rate of growth of Bact. coli

MEDIUM	pH	TIME REQUIRED TO SHOW TURBIDITY
		hours
1 per cent pepton	Approx. 7.0	5
1 per cent pepton 0.20 M NaCl	" 7.0	$3\frac{1}{2}$
1 per cent pepton 0.20 M KCl	" 7.0	$3\frac{3}{4}$
1 per cent pepton 0.20 M NH_4Cl	" 7.0	$3\frac{3}{4}$
1 per cent pepton 0.20 M $MgCl_2$	" 7.0	8
1 per cent pepton 0.20 M $CaCl_2$	" 7.0	120
1 per cent pepton 0.20 M $FeCl_3$	" 7.0	No growth

The results are shown in table 3. $MgCl_2$ and NaCl, therefore, in concentrations where the number of Cl ions is the same, are comparable in effect. That there is a cation effect in greater concentrations, however, is shown by the fact that the time for 0.20 molar $MgCl_2$ is 12 hours, while that for 0.40 molar NaCl is but $4\frac{3}{4}$. Doubling the NaCl concentration changes the time rate very little, while doubling the $MgCl_2$ concentration more than triples the time. This is strong evidence that there is a cation effect, though it may not be so marked as the anion effects. $CaCl_2$ has a much stronger inhibiting effect while $FeCl_3$ entirely inhibited growth in the concentration used.

Our results so far have been obtained upon media adjusted to a pH of approximately 7.0. Since we know that the H-ion concentration materially affects growth of bacteria, it is of both interest and value to know to what extent the salts modify the time element at pH values on either side of neutrality. Table 4 shows these effects with the salts given and at the H-ion concentrations stated. The results indicate that the different salts

TABLE 3

Showing the effects of various concentrations of NaCl and of $MgCl_2$ upon the rate of growth of Bact. coli

MEDIUM	pH	TIME REQUIRED TO SHOW TURBIDITY
		hours
1 per cent pepton 0.20 M NaCl................	7.0	$3\frac{1}{4}$
1 per cent pepton 0.40 M NaCl................	7.0	$4\frac{3}{4}$
1 per cent pepton 0.10 M $MgCl_2$................	7.0	$3\frac{1}{4}$
1 per cent pepton 0.20 M $MgCl_2$................	7.0	12

TABLE 4

Showing the effects of various salts upon the growth of Bact. coli at different H-ion concentrations

MEDIUM	TIME OF VISIBLE GROWTH AT pH VALUES OF				
	5.2	6.2	7.6	8.2	9.2
	hours	*hours*	*hours*	*hours*	*hours*
1 per cent pepton............................	$17\frac{1}{4}$	$5\frac{3}{4}$	$6\frac{1}{2}$	8	32
1 per cent pepton 0.20 M NaCl................	$5\frac{1}{2}$	$4\frac{1}{2}$	$3\frac{1}{2}$	3	14
1 per cent pepton 0.20 M Na_2SO_4..............	$6\frac{1}{2}$	$4\frac{1}{2}$	$4\frac{1}{4}$	$3\frac{1}{2}$	
1 per cent pepton 0.20 M Na citrate..........	26	$8\frac{1}{2}$	$7\frac{1}{2}$	22	

have marked changes of effect with changes in pH. In general we may say that NaCl and Na_2SO_4 widen the optimum range of growth, while Na citrate narrows this pH range.

DISCUSSION

The data presented show in a general way some correlation between the so-called lyotropic series and the order of effect upon the growth of *Bact. coli.* There are, however, ions which are exceptions and which, in concentrations thus far tried, have proved highly retarding in their action. These ions are the sulphocyanate and fluoride. Whether in lower concentrations they might not prove but slightly retarding or even beneficial to growth remains to be ascertained. The position of the SO_4 radical with regard to effect upon bacterial growth is also somewhat at variance with its usual position in the lyotropic series. Instead of being found opposite the iodine end of the series it is found next to the Cl and I radicals.

While these are deviations from the usual order, it is not surprising since in many of the phenomena in biochemistry the lyotropic order does not strictly compare with the usual order as determined by effects upon surface tension, viscosity, etc., especially at different concentrations and temperatures. We must also remember that here we are dealing with an added factor which is not present with proteins in solution or with colloids in general; that is, the life of an organism. Since this is our measure of effects it must be taken into account. We do not know what properties affect viability most, and consequently we have no means of knowing what mechanism causes retarding and inhibitory effects. There seems to be, as might be expected, an order of specificity which must be taken into account.

The general order of the lyotropic series, however, holds at pH 7.0 Cl and I are found at one end of the series aiding or accelerating action, while the citrate, acetate, and oxalate at the other end retard growth.

Considering the effect of cations, we have a close analogy between action here and action of salts in the animal body. As might be expected, there is little difference between the Na, K, and NH_4 ions. With Mg the action begins to manifest itself, although it is greatly modified by the anion effect. The calcium ion produces its characteristic strong effect.

It is in media of different H-ion concentrations that these effects become significant. In the region of optimum growth the influences are not exceedingly marked, but as we near the H-ion concentrations which mark the limits for growth of *Bact. coli* the differences in the rate of growth are greatly increased. In other words the H-ion range for optimum growth is widened or narrowed as shown in the table given.

This factor becomes of practical value in adjusting media for optimum bacterial growth. The figures show that certain ions are of value in pepton media for accelerating the growth of *Bact. coli* and also for widening the range for optimum growth, while certain other ions narrow the H-ion range and decrease the rate of growth. This would perhaps explain the findings of Cohen and Clark (1919) that culture media adjusted with HCl had a higher limit of growth on the acid side than media adjusted with acetic acid.

SUMMARY.

It has been shown that the growth of *Bact. coli* in 1 per cent pepton medium is accelerated or retarded by different salts in low molecular concentrations.

The salt effects at various H-ion concentrations vary greatly. Those salts which accelerate growth seem to widen the H-ion range for optimum growth, while those which retard growth seem to narrow the limits for optimum activity.

Cations and anions are both effective.

REFERENCES

Bechhold, H., and Ziegler, J. 1919 *In* Colloids in biology and medicine, by H. Bechhold and J. G. M. Bullowa, p. 55.

Brooks, M. M. 1919 Jour. Gen Physiol., **2**, 5.

Cohen, B., and Clark, W. M. 1919 Jour. Bact., **4**, 409.

Greaves, J. E. 1916 Soil Science, **2**, 443.

Gustafson, F. G. 1919 Jour. Gen. Physiol., **2**, 17.

Loeb, J. 1918–1921 Jour. Gen. Physiol., 1918–19, **1**, 39, 237, 363, 483, 559; 1919–20, **2**, 87, 273; 1920–21, **3**, 85, 247, 391.

Mathews, A. P. 1906 Jour. Infect. Dis., **3**, 572.

Winslow, C.-E. A., and Falk, I. S. 1919 Abs. Bact., **3**, 5.