

EXPERIMENTS ON THE ADAPTATION OF *ESCHERICHIA COLI* TO SODIUM CHLORIDE

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INTRODUCTION

Since the isolation and pure culture study of microorganisms, investigations on the variability and adaptability of this biological material have frequently been undertaken with the hope of thereby contributing to some fundamental biological problems. A particular impetus to such studies was given by the development of the theory of evolution, the controversy between the monomorphistic and pleomorphistic viewpoints, and, more recently, by the discovery of adaptive enzyme formation. It seemed likely that an examination of the behavior of the outwardly undifferentiated microorganisms could contribute much towards a better understanding of the underlying principles of the phylogenetic evolution of higher forms.

While some definite results and interpretations have been obtained in studies on protozoa (Jollos (1), Jennings (2, 3), Hoare (4), Chatton and Tellier (5)), similar investigations with bacteria have yielded less definite, and sometimes controversial data. The reasons for this are not difficult to understand. Protozoa can be studied even as single individuals, which is virtually impossible in the case of bacteria. The experiments on the latter have, therefore, dealt with progenies of individuals rather than with the units themselves, so that the interpretations must be based on statistical analyses of growth curves, the morphological aspects of colonies, etc. Yet, the recent developments in the study of microbic variations (Beijerinck (6), Hadley (7), Mayer (8), Pinner and Voldrich (9), Doudoroff (10-12)) have clearly demonstrated that individuals in a pure culture may possess morphologically, physiologically, and biochemically different characteristics which can be transmitted to their offspring. On the other hand, the most important investigations on adaptation (Kluyver and Baars (13), Burke and coworkers (14, 15), Vaas (16), Karström (17), Stephenson and coworkers (18, 19), Knight (20), Lewis (21)) have not always made it possible to decide how far the observed variations were caused by the environment through a direct modification of the cells or through selection of individuals with certain pre-existing potentialities.

A serious difficulty in the application of the results of such studies to the interpretation of adaptability, mutability, and evolution of higher forms is the apparent absence of sexual phenomena in many of the microorganisms.

A detailed survey of the literature pertaining to these problems is found in the recent study of Vaas on adaptation of *Bacillus megatherium* to sodium chloride (16), to which the reader is referred.

The following report represents an exploratory study of the adaptation of *Escherichia coli* to an unfavorable environment. Sodium chloride was used in preference to other inhibitory agents because of the ease of its administration and the considerable literature on halo-tolerance and adaptation to saline media.

I

*Preliminary Experiments and Standardization of Methods**1. Preliminary Experiments*

The first experiments were designed to study the behavior of bacteria grown in ordinary fresh-water media on transfer to unfavorably saline nutrient solutions. The results can here be briefly summarized since Vaas (16) has recently published experimental data which are in complete agreement with my own. With an increase in the salinity of the medium the viable count of various fresh-water bacteria¹ remains constant until a certain NaCl concentration, varying with the species, has been reached; thereafter this count drops progressively with further increase. These results were interpreted to mean that a selective process was involved, some cells being better able to survive and reproduce under unfavorable conditions than others. The proportion of cells capable of reproducing in a medium with a certain salt concentration was fairly constant under a given set of conditions. This suggested a means of studying the "acclimatization" process, using as a criterion of adaptive changes the ability of the organisms to reproduce under unfavorable conditions, as measured by changes in viable counts made in defined saline media. This criterion of acclimatization is essentially the "success test" of animal and plant ecologists, described by Shelford.² A few investigators, *e.g.* Chatton and Tellier (5), and Vaas (16), have recognized the advantages of this criterion over those more commonly applied but basically different, such as (1) the death point or death time of organisms subjected to lethal concentrations or doses of the factors to which they had been acclimatized (Jacobs (23), Jollos (1),

¹The bacteria used were: *Bacillus niger*, *Bacillus subtilis*, a species of *Pseudomonas* isolated from enrichment cultures containing inorganic salts and ethylene glycol, and two strains of *Escherichia coli*.

²Shelford, V. E., Laboratory and field ecology, Baltimore, The Williams & Wilkins Co., 1929, 99.

etc.), and (2) the metabolic rate of organisms in an unusual or unfavorable environment (Kluyver and Baars (13), Stephenson and coworkers (18, 19)).

Our method depends on the assumption that cells capable of reproducing in saline media produce viable offspring, and can therefore be counted by the colony or dilution method technique. The fairness of this assumption has been demonstrated by the results reported by Vaas (16) as well as my own.

It was necessary to standardize a technique for determining the number of bacteria viable in various concentrations of salt so as to obtain constant and reproducible results. Plate counts were found entirely unsatisfactory when high concentrations of salt were used in the agar. After some research the use of plate counts was abandoned, except for determinations of viable counts with little or no salt, and instead, the much more tedious, and under ordinary conditions less accurate method of broth dilutions was adopted. (Cf. (24), (25), and (26^a)). Counts obtained in fresh-water broth agreed very closely with those arrived at by the plate method, and, as evidenced by the various tables, this method gave satisfactory results also when saline broth was used. Although the absolute viable counts in the same salt concentration varied from experiment to experiment, duplicate counts from the same culture were always in satisfactory agreement. The significant changes in viable counts induced experimentally were far greater than the variation among comparable counts in duplicate experiments. An additional advantage of the dilution method is the fact that the counts are made under conditions more closely approximating those of the cultures in which the organisms were grown.

For the development of a "standard technique" it was necessary to determine the effect of factors which influence considerably the viability of bacteria in saline broth.

The complexity of the problem soon made it necessary to limit either the scope of the investigation or the number of organisms to be used. Inasmuch as it was expected that a more comprehensive study of a single strain would yield more fruitful results, the experiments discussed in the following sections were carried out with a strain of *Escherichia coli*. This organism was chosen because so much work has been published dealing with its physiology. It should, however, be pointed out that a number of other organisms may have advantages for the study of such adaptive processes which would tend to weaken the justification for the continued use of this common intestinal

^a Buchanan, R. E., and Fulmer, E. I., Physiology and biochemistry of bacteria, Baltimore, The Williams & Wilkins Co., 1928, 1, 10.

commensal, whose chief claim to popularity seems to be its abundant occurrence in feces, sewage, and drinking water. The strain used was a stock culture, isolated at the Hopkins Marine Station some 3 years previously, and kept since on yeast agar slants.

2. Hydrogen Ion Concentration

The pH of the saline broth in which the dilution counts were made is an important factor affecting the viability of the organisms. (See Table I.) For this reason the pH of the medium was carefully controlled and a considerable amount of buffer used.

The results summarized in Table I were obtained by making dilution counts of a suspension from a 24 hour yeast extract culture in media with

TABLE I
Dilution Counts of a Culture of E. coli in Media with Different NaCl Concentrations at Various pH Values

Viable counts with.....	0 per cent NaCl	5 per cent NaCl	6 per cent NaCl
pH			
6.0		4.5×10^4	2.5×10^4
6.5		2.0×10^6	9.5×10^5
7.0	1.8×10^9	7.5×10^7	4.5×10^8
7.5		9.5×10^7	2.5×10^8
8.0		6.5×10^7	4.5×10^8

two different NaCl concentrations, and adjusted to various hydrogen ion concentrations with the aid of a glass electrode.⁴

An acid reaction of the medium combined with high salinity is unfavorable to growth. Sherman and Holm (32) showed that NaCl increases the tolerance of *E. coli* to hydrogen ions. This increased tolerance of microorganisms to various unfavorable influences in the presence of relatively low salt concentrations may be rather general (Baars (28), van Niel (29)). Yet, the results laid down in Table I are not in conflict with these observa-

⁴The general composition of the nutrient broth was as follows:

Distilled water

Yeast autolysate..... 2.5 per cent by volume

KH_2PO_4 0.135 per cent

$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ 0.78 per cent

NaCl..... 5 per cent and 6 per cent respectively

NaOH or HCl to desired pH

The yeast autolysate was prepared according to the method described by Elema ((27), p. 80).

tions; the salt concentration was so great that in itself it exhibited a toxic effect. The additional burden of an unfavorable hydrogen ion concentration thus merely increased the inhibition.

Between pH 7 and 8 there was no significant difference in viable counts. Hence neutral media were used for subsequent experiments, since the buffering capacity of the phosphates is greater there than at higher pH values, and since the cultures in yeast extract become alkaline as a result of metabolism.

3. Concentration of Nutrients

Table II shows that in media with 6 per cent and 7 per cent NaCl more bacteria could develop if the concentration of nutrients was reduced.

TABLE II
Number of Bacteria Developing in Saline Broth with Different Concentrations of Yeast Autolysate

Viable counts with.....	6 per cent NaCl	7 per cent NaCl
Concentration of yeast autolysate in per cent by volume		
0.5	2.5×10^8	4.5×10^8
1.0	7.5×10^8	2.5×10^8
2.5	4.5×10^8	9.5×10^8
5.0	4.5×10^8	2.5×10^8
10.0	2.5×10^8	7.5×10^8

These surprising results would lead one to believe that in the presence of high concentrations of NaCl the yeast autolysate exerts an additional inhibitory influence. The possibility seems excluded that such an effect would be due merely to an increase in the total osmotic pressure of the solution. From the observed boiling point of undiluted yeast autolysate the concentration of dissolved substances was computed to correspond osmotically to less than 2 per cent NaCl. The difference in osmotic effect between 0.5 and 1 per cent yeast autolysate would thus amount to an increase corresponding to only 0.01 per cent NaCl. The counts in 2.5 per cent yeast autolysate with 6 per cent NaCl and in 0.5 per cent yeast autolysate with 7 per cent NaCl show that the number of viable organisms in these two media is the same. Yet, the increase in osmotic effect due to the difference in yeast autolysate concentration corresponds to at most 0.04 per cent NaCl. Also, in media with 6 per cent NaCl an increase in nutrient concentration from 2.5 per cent to 5 per cent causes no further drop in the

viable count, while in the presence of 7 per cent NaCl the same difference in yeast autolysate concentration results in a 40-fold decrease. If one then considers that the number of viable organisms in 7 per cent NaCl-0.5 per cent yeast autolysate decreases to about 0.002 of the original with an increase of the yeast autolysate concentration to 2.5 per cent, whereas a further doubling of the amount of nutrients in 6 per cent NaCl has no effect, it becomes apparent that the influence of the concentration of yeast autolysate on the viability of the bacteria in NaCl media is quite complex.

No further attempts were made to elucidate this situation, and for later experiments a yeast autolysate concentration of 2.5 per cent was chosen arbitrarily. It may seem that a lower concentration would have been advantageous; yet at such concentrations the total growth of the bacteria decreases considerably, and even the gross results become inconsistent, varying with the batch of yeast autolysate used.

4. *Aeration*

Observations on the reproduction of bacteria taken from a fresh-water medium and suddenly immersed in saline broth showed that aeration also is an important factor in determining how many individuals will reproduce in the unfavorable environment. (See also (30, 31).)

Dilution counts of bacteria from a 24 hour yeast extract culture were made in 7 per cent NaCl broth under the following conditions:

1. In test tubes slightly stirred to insure an even suspension of organisms only.
2. In test tubes vigorously stirred after inoculation, and then left undisturbed.
3. In bottles, providing a large surface of medium exposed to the air.
4. In similar bottles, constantly rotated to insure thorough agitation of the medium.
5. In test tubes, slightly stirred, as in (1), the contents of which were placed in bottles after 24 hours of incubation and constantly rotated as in (4) till the completion of the experiment.
6. In bottles, constantly rotated as in (4) for 6 hours, after which time the contents were transferred to test tubes, and left unagitated as in (1).

The results are shown in Table III.

It is apparent that aeration during the first few hours after inoculation considerably increases the number of bacteria capable of reproducing in the saline medium.

For viable counts in further experiments, where at times several hundred cultures had to be incubated simultaneously, it was impractical to use constant stirring, and we were limited to the use of test tubes. The tubes were stirred vigorously for 10 seconds after inoculation and then left undisturbed. Although maximum values would not be obtained with this

method, the expectation that comparable results would be obtained by the use of a uniform technique was proved to be correct by further tests.

5. Standard Method

The standard method adopted for the further experiments may be summarized as follows:

1. All culture media were made with distilled water, and contained 0.5 per cent by weight KH_2PO_4 - Na_2HPO_4 Sørensen buffer at pH 7 and 2.5 per cent yeast autolysate by volume (27). When salt was used in the media, sufficient NaOH was added to bring the pH to 7.0-7.1 as determined with the glass electrode.
2. Ordinary viable counts were made on yeast agar plates (10 per cent yeast autolysate in tap-water with 2 per cent agar, 0.1 per cent K_2HPO_4 , and 0.05 per cent MgSO_4). In some cases, 3 per cent NaCl was added to the agar (where indicated). This salinity

TABLE III
Aeration and Viability of E. coli in Saline Broth

	Viable count in 7 per cent NaCl broth
1. In tubes, not vigorously stirred.....	4.5×10^4
2. In tubes, stirred after inoculation.....	6.5×10^8
3. In bottles, not stirred.....	1.5×10^8
4. In bottles, constantly agitated.....	2.5×10^8
5. In tubes during 24 hrs., then agitated in bottles.....	7.5×10^4
6. Agitated in bottles for 6 hrs., then transferred to tubes.....	2.5×10^8

did not reduce the viable count when organisms were taken from a fresh-water medium (see Table IV) and was used in those cases where the bacteria had been grown in more concentrated saline broth and a sudden change to fresh-water medium was not desired.

3. Total cell counts were made with a Petroff-Hausser counting chamber.

4. Viable counts in saline broth were made by the dilution method. Ordinarily, three series of decimal dilutions of the culture in saline yeast extract medium were prepared. The tubes were thoroughly shaken for 10 seconds after inoculation and sealed with "parafilm" to prevent concentration of the solution by evaporation. The most probable number of viable cells was determined from the tables prepared by McCrady (25).

5. The cultures from which the viable counts were made were grown in aeration flasks; the air bubbled through the cultures was passed through a column of NaCl solution at the same temperature and of the same salinity as that of the cultures.

6. The initial inoculum for an experiment was standardized by making two successive transfers in aerated fresh-water broth, each incubated for 24 hours.

7. All cultures were grown at 35°C.

By using this standardized technique, the results yielded by duplicate determinations in any one experiment agreed quite closely. The following

experiment demonstrates this. A 24 hour fresh-water broth culture was used for the estimation of the number of cells viable in a fresh-water and a saline medium. Six independent determinations were made, each consisting of three series of dilutions in 6 per cent NaCl broth. The results showed a total viable count (fresh-water medium) of 2.4×10^9 organisms per cubic centimeter and 2.5×10^5 , 2.5×10^5 , 4.0×10^5 , 4.5×10^5 , 4.5×10^5 , 6.5×10^5 bacteria per cubic centimeter viable in saline broth.

In view of the fact that the dilution method *per se* is considerably less accurate than the plate count method, and that the viability of *E. coli* in 6 per cent NaCl media is dependent upon so many factors, the individual variations are not surprising, and the results may be deemed satisfactory.

The agreement between viable counts obtained by the dilution and plate methods in fresh-water media, and in those containing 3 per cent NaCl is

TABLE IV
Comparison between Plate Counts and Dilution Counts without and with 3 per cent NaCl

	Exp. 1	Exp. 2
Total cell count (determined microscopically).....	2.7×10^9	2.1×10^9
Viable count, plate method, no NaCl.....	2.4×10^9	1.9×10^9
“ “ dilution “ “ “	2.5×10^9	2.0×10^9
“ “ plate “ 3 per cent NaCl.....	2.2×10^9	1.9×10^9
“ “ dilution “ “ “	2.0×10^9	2.5×10^9

revealed by Table IV, representing two experiments performed on different days.

It appears that the variation between duplicate experiments is insignificant at low concentrations of salt. It becomes, however, progressively wider with the use of more concentrated saline broth, as may be seen in Table V and in subsequent experiments.

II

Individual Variation in Adaptability to Saline Media

1. Differential Counts in Various Salt Concentrations

It has been stated that the proportion of individuals in a fresh-water culture capable of reproducing in a saline environment becomes progressively smaller with an increase in salinity. Table V is presented in illustration of this phenomenon.

This relationship between the percentage of viable organisms and the salinity of the medium was practically constant in a large number of experi-

ments, provided that the bacteria were grown in fresh-water broth under standard conditions. The experiments discussed in the following pages were designed to study the various factors by which this relationship can be changed.

2. Influence of the Developmental Phase of the Culture

Vaas (16) has shown that the ability of *B. megatherium* to grow in saline media, as determined by the fraction of total viable cells in fresh-water medium able to produce colonies in saline agar, is a function of the developmental stage of the culture. The adaptability was lowest in the early logarithmic period and greatest during the early stationary phase.⁵

TABLE V
Relation of Viable Count to NaCl Concentration

NaCl per cent	Viable count	
	Exp. 1	Exp. 2
0	2.5×10^9	2.0×10^9
3	2.0×10^9	2.5×10^9
4	9.5×10^8	4.5×10^8
5	2.5×10^7	7.5×10^6
6	4.5×10^6	2.5×10^4
7	9.5×10^2	2.5×10^1
8	Less than 1×10^0	

To determine the relation between the "physiological state" and the viable counts in saline broth, 100 cc. of 2.5 per cent yeast autolysate broth were inoculated with 0.001 cc. of a 24 hour culture of *E. coli*. Samples were taken out after various intervals of time. Total cell counts and total viable counts on yeast agar plates were made, as well as dilution counts in 5 per cent and 6 per cent NaCl broth. Fig. 1 shows the composite curves for three such experiments.

The total cell counts agreed fairly closely with the total viable counts in fresh-water media for the first 24 hours. Thereafter, the total cell count increased slowly, becoming about 15 per cent higher at 48 than at 24 hours; the viable count remained constant. The reaction of the culture was neutral during the first 8 hours of incubation, then became alkaline, the pH being 7.3 after 24, and 7.8 after 168 hours.

The results obtained with *E. coli* are in perfect agreement with the findings of Vaas for *B. megatherium*. As judged by the ratios of viable counts in saline broth to those in fresh-water media, the ability of *E. coli* to grow in a saline environment is greatest during the early stationary and least

⁵ For the terminology used see Henrici (33).

during the logarithmic phase. Since the cultures were started with organisms in the early stationary phase, or at the peak of adaptability, the ratio decreases during the so called "lag" period. Adaptability decreases with senescence of the culture.

The curves bear a striking similarity to those obtained for the thermal death of *Paramecium* (Doudoroff (34)), and show the same relation of the physiological condition of the cultures to the adaptability of the organisms as to their resistance to lethal agents, as shown particularly by Sherman and Albus (35), and by Robertson (36). It is now generally recognized that organisms are most susceptible to toxic agents during the logarithmic phase of development. That such "physiologically young cells" (Sherman and Albus) can least stand transfer to unfavorable conditions suggests an

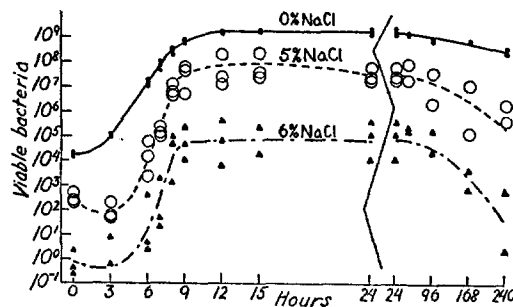


FIG. 1. Influence of the developmental phase of the culture on the adaptability of *E. coli* to sodium chloride.

a medium of given salinity. Growth in a concentrated saline environment involved a selection of those bacteria capable of reproducing under such conditions. That another factor besides the physiological state of the culture could be responsible for significant changes in the absolute value of this fraction soon became apparent.

If the salinity of the medium was raised gradually, more organisms from a 24 hour (stationary phase) culture were viable in saline broth than if the bacteria were suddenly immersed in the unfavorable medium. This agrees with numerous observations on the acclimatization of microorganisms to unfavorable environments by subjecting them to small doses of the toxic agent as reported in the literature (23, 5, 14, 15).

Equal portions of a 24 hour fresh-water culture were placed in each of four tubes and constantly aerated. At regular intervals concentrated NaCl solution (13.5 per cent NaCl, 0.5 per cent buffer) was added to each tube in such amounts as to increase the salinity of the mixture by 1 per cent NaCl steps with each addition. The intervals

intimate relation between their ability to reproduce under these conditions and their resistance to sublethal doses of toxic agents as established by the usual criteria of death.

3. Selection or Acclimatization?

It has been shown that a certain fraction of the total number of bacteria in a fresh-water culture could adapt themselves to an existence in

between additions were 15, 30, 60, and 120 minutes in the four tubes respectively. In this manner, the salt concentration in the tubes was increased by steps of equal magnitude but of different duration. Viable counts were made in broth containing various concentrations of salt (from 3 per cent to 8 per cent). The samples for these dilution counts were taken after the organisms had been subjected to a NaCl concentration 1 per cent lower than that of the final test medium for a period of time corresponding to the duration of one step. Thus, the counts in 8 per cent NaCl broth were made 15, 30, 60, and 120 minutes after the seventh addition of salt to the tubes. Counts were likewise made using sudden immersion from the original fresh-water culture, corresponding to steps of no duration, and on salt-free agar plates.

The results are presented in Fig. 2. In Fig. 2A the number of bacteria viable in various saline media is plotted against the duration of each inter-

mediate step, while in Fig. 2B the same data are entered with the viable count as ordinate and the concentration of salt as abscissa. It will be noted that all organisms were viable if placed directly into 3 per cent NaCl broth. Less and less cells could survive sudden transfer to more concentrated media. However, when the organisms were gradually subjected to the effect of higher salt concentrations greater numbers could reproduce in the unfavorable environment.

That this acclimatization, as expressed by changes in the developing fraction of bacteria, was a process of individual adaptation of the cells, in no way dependent on growth or selection, appears from the following considerations. The production of new cells would not be expected in a culture in the stationary phase when such a culture is subjected to unfavorable conditions without any addition of nutrients. No reproduction could be observed in the aeration tubes, either by plate counts, total cell counts, or determinations of the total volume of bacterial mass. If it is kept in mind that the initial discrepancy between the viable counts in fresh-water and in 4 per cent NaCl media disappeared as a result of the gradual increase in salinity, it becomes clear that, had this behavior been due to the formation of new cells in the mother culture, an increase in numbers would have

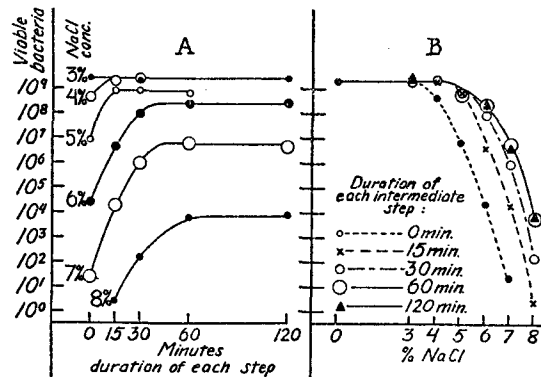


FIG. 2. Acclimatization of non-reproducing individuals of *E. coli* to sodium chloride by subjection to regularly increasing salt concentrations.

been readily detectable. Hence it seems that the gradual addition of salt has an effect on all rather than on some of the cells, and the increase in the fraction of the cells capable of reproducing in saline broth is merely an index of this effect, as measured by the hardiest or most "adaptable" individuals.

Fig. 2 shows further that the length of exposure to each concentration is of paramount importance. A maximum adaptation is obtained only by using exposures of sufficient duration. Shorter "steps" give lower values, while longer ones do not change the results of the viable counts. The minimum length of step giving maximum acclimatization depends on the final concentration in which the counts are made; the greater the salinity, the longer must be the duration of the intermediate exposures. Since the successive intermediate steps in each series were all of the same duration, it is impossible to determine whether the first or any of the later ones were responsible for the observed minimum period.

This type of acclimatization differs materially from the "race adaptation" brought about by the selection and perpetuation of those organisms capable of surviving, although in this case it has been detected and measured by the changes in the latter. It seems to be similar to the adaptation to salt in the protozoan, *Glaucoma piriformis* (5), and perhaps to the so called acclimatization to lethal temperatures in *Paramecium* (23).

III

Factors Influencing Non-Selective Acclimatization

1. Influence of Intermediate Salt Concentration

Fulmer (26)⁶ as well as Chatton and Tellier (5) have shown that in order to induce acclimatization an exposure of the organisms to a single intermediate concentration of salts may be substituted for exposure to a number of small steps. It seemed desirable to find a single intermediate concentration of NaCl which would have an effect similar to that of a gradual increase in salinity.

Equal quantities of a 24 hour culture of *E. coli* grown in the absence of salt were mixed with equal volumes of saline solutions containing 0.5 per cent buffer at pH 7 and sufficient NaCl to bring the salinity of the cultures to 0, 0.5, 1.0, 1.5, 2.5, 3.5, 4.5, 5.5, and 6.5 per cent in the various tubes respectively. The mixtures were continuously aerated at 35°C. Samples were taken after various periods of time and counts made in 6.5 per cent NaCl broth. In those cases where the viable count in saline broth increased over that before the addition of salt, it was again found to reach a maximum after a short period of time.

⁶ Buchanan, R. E., and Fulmer, E. I., *Physiology and biochemistry of bacteria*, Baltimore, The Williams & Wilkins Co., 1928, 2, 223.

The relation between the size of the intermediate step and the maximum number of organisms viable in 6.5 per cent NaCl broth at any time after the addition of salt is shown in Fig. 3. No reproduction of the bacteria could be detected.

As might be expected, no difference in adaptability of the organisms was observed among those receiving buffer only, those immediately brought up to the full concentration of the test medium (6.5 per cent NaCl), and those suddenly immersed in the saline broth without any intermediate treatment. Those bacteria, however, exposed to different salinities showed varying degrees of acclimatization, depending on the magnitude of the

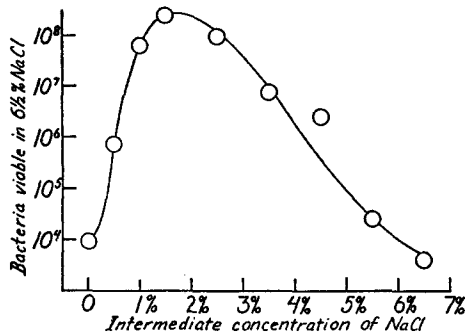


FIG. 3

FIG. 3. Acclimatization of *E. coli* to sodium chloride by subjection to a single intermediate salt concentration. Relation between the magnitude of the first step and the maximum number of bacteria becoming viable in 6.5 per cent NaCl broth.

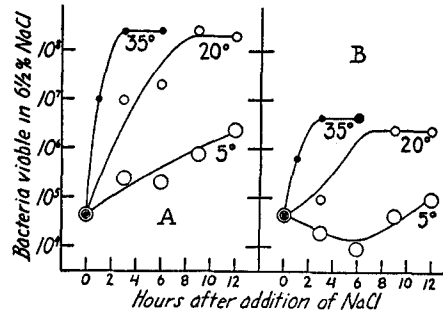


FIG. 4

FIG. 4. Effect of temperature on individual acclimatization.

A. 2.5 per cent intermediate NaCl concentration.

B. 4.5 per cent intermediate NaCl concentration.

intermediate concentration. It may be judged from this that the increase in viable counts was truly elicited by the salt and was not an artefact due to the dilution of the medium, aeration in saline medium, or some other such factor incidental to the addition of salt.

It appears that an intermediate step of about 1.5 per cent NaCl results in an optimal acclimatization. Very low concentrations are insufficient to cause maximal adaptation, while very large ones seem to be nearly as injurious to the bacteria as the saline broth in which the counts were made.

2. Effect of Age of the Culture

Old cultures were not as readily acclimatized to saline environment by the gradual addition of salt as were cultures in the early stationary phase of development.

Three samples were taken from a culture in the stationary phase 24 hours after inoculation, three after 48, and three after 96 hours. One sample from each period was used for immediate dilution counts in 6.5 per cent NaCl broth without previous treatment; the second and third samples received sufficient 13.5 per cent NaCl to bring the salt concentrations to 1.5 per cent and 4.5 per cent respectively. Portions of these were tested for viability in 6.5 per cent NaCl broth after exposure for varying lengths of time to these intermediate steps. The results are shown in Table VI.

It will be seen that as the culture ages and the number of bacteria capable of immediate reproduction in a saline medium decreases, the maximum number capable of being acclimatized also decreases. While in the case of a small intermediate concentration this decrease is comparable to that observed for the unacclimatized bacteria, in the case of a larger and less favorable intermediate step, the decrease is more pronounced. This sug-

TABLE VI
Effect of Aging on Acclimatization

Age of culture <i>hrs.</i>	Maximum viable count in 6.5 per cent NaCl broth		
	Intermediate concentration		
	None	1.5 per cent NaCl	4.5 per cent NaCl
24	9.5×10^8	2.0×10^8	4.5×10^6
48	4.5×10^8	7.5×10^6	4.5×10^3
96	9.5×10^0	7.5×10^4	2.0×10^1

gests that increased susceptibility to injury by salt may be an earlier consequence of aging than the loss of "adaptive power" and may, in fact, be the cause of the latter.

3. *Effect of Temperature*

The rate of "individual acclimatization" depends on the temperature at which the organisms are subjected to increasing salt concentrations.

Portions of a 24 hour salt-free culture were introduced into aeration tubes, at 5°, 20°, and 35°C. respectively. Sufficient 13.5 per cent NaCl solution containing 0.5 per cent buffer was added to bring one set of cultures at the three temperatures to 2.5 per cent NaCl and the other set to 4.5 per cent NaCl.

After various intervals of time samples were taken from each tube and dilution counts made in 6.5 per cent NaCl broth. The results are shown graphically in Fig. 4, the data obtained with 2.5 per cent and 4.5 per cent intermediate concentrations being presented in parts A and B respectively.

Although the theoretical and practical limitations of the method used for determining the rate of adaptation made it impossible to plot the curves

accurately, it is apparent that acclimatization occurs more rapidly at higher temperatures than at lower ones.

Insufficiency of data and the difficulty of interpreting the results made the determination of a temperature coefficient impossible, although from the mere inspection of the curves a Q_{10} approximating 2 may be estimated. The difference in rates at different temperatures might be due to the combination of several factors responsible for "adaptive processes" on the one hand, and death of the bacteria on the other. This might explain the differences in the curves obtained with the more or less "optimal" intermediate salt concentration of 2.5 per cent and the less favorable one of 4.5 per cent.

TABLE VII
Reversibility of Acclimatization

	Time	Bacteria viable in 6.5 per cent NaCl broth	
	<i>hrs.</i>		
Before acclimatization.....		9.5×10^8	
After acclimatization.....	0	2.5×10^8	
		Bacteria resuspended in buffer	Bacteria resuspended in 1.5 per cent NaCl
"	1	2.5×10^8	7.5×10^7
"	3	4.5×10^4	2.5×10^8
"	6	2.0×10^8	9.5×10^7
"	20	4.5×10^4	2.5×10^8

A similar effect of temperature was demonstrated when adaptation was induced by gradually increasing the salt concentration.

4. *Reversibility of the Process*

Samples from a 24 hour culture in NaCl-free medium were subjected to 1.5 per cent NaCl for 4 hours, at which time the number of organisms capable of reproducing in 6.5 per cent NaCl had reached a maximum. A part of this culture was diluted with nine parts of distilled water containing 0.5 per cent buffer only. As a control, a similar portion was diluted with nine parts of buffered 1.5 per cent salt solution. Both suspensions were aerated at 35°C., and samples taken from each after various intervals of time for dilution counts in 6.5 per cent NaCl broth. No reproduction could be detected in the two suspensions either by plate counts or by determinations of the volume of bacterial mass. Table VII shows the results.

Within a few hours after the acclimatized organisms had been returned to fresh water, they had completely lost their increased ability to develop in saline media, as judged from the fact that the number of bacteria viable in 6.5 per cent saline broth had dropped below the original value obtained

before the conditioning process. At the same time those organisms remaining in 1.5 per cent NaCl solution showed little if any change in adaptive power. The relationship is even more striking after 20 hours, when in both cases a decrease in viable count in saline broth was observed. This decrease may be the result of a rapid senescence of the bacteria caused by the increased salinity combined with the dilution of the nutrients in the medium. It seems that such senescence would proceed equally rapidly in fresh-water and in 1.5 per cent NaCl solutions; the relative decrease in viable counts in the two media is practically the same between 6 and 20 hours. It thus appears that the processes responsible for the individual acclimatization are readily reversible.

That the decrease in the number of salt-viable bacteria is not due to death of the organisms is shown by the fact that the counts on salt-free agar not only failed to show an increase, but also revealed no significant decrease in the number of bacteria viable in fresh-water media. Thus, whereas the number of living individuals in the 1.5 per cent NaCl and in the NaCl-free solutions was the same, it was only in the former that they retained their ability to develop in the strongly saline medium. This, after all, is equivalent to stating that the process of individual acclimatization is reversible.

IV

Characteristics of Cultures Developing in Saline Media

1. Storage of Acclimatized Bacteria in Saline Broth

The experiments reported in the foregoing sections have dealt with the behavior of *E. coli* in salt-water media when the bacteria had been grown in NaCl-free environments, but without growth during the acclimatization period.

Before studying the effects of growth in a saline medium on adaptation it seemed desirable to determine what changes in adaptability might occur in organisms acclimatized to salt broth and left in this medium for a considerable period of time without opportunity for reproduction.

A 24 hour fresh-water culture was gradually brought up to 7 per cent salinity by seven 1 per cent steps of 1 hour duration, thus assuring maximum acclimatization of the bacteria to the saline environment. The resulting suspension was constantly aerated at 35°C. for 6 days and dilution counts in 7 per cent and 8 per cent NaCl broth, as well as plate counts on 3 per cent NaCl yeast agar were made after various intervals of time following the acclimatization. The results of two such experiments are plotted in Fig. 5A.

It is apparent that organisms acclimatized by the gradual increase in salinity remain in this state for a considerable period of time. No further significant increase in viable count in saline broth beyond the initially established maximum occurs. The curves also demonstrate that a part of the bacteria must have died in the salt medium, because the number of organisms viable in 3 per cent NaCl dropped somewhat, particularly during the early stages of the experiment. At the same time, the viable count in 7 per cent NaCl broth remained remarkably constant; no indication of a similar initial decrease was observed. The two curves thus show that the individuals which had become fully adapted are less readily killed.

Although the non-adapted cells die more rapidly than the adapted ones, yet the death rate is comparatively low. Hence, at all times the medium contains organisms incapable of reproducing in 7 per cent NaCl solutions but capable of development under more favorable circumstances, such as in a 3 per cent salt medium. These considerations show that there exists a real difference between the general physiological characteristics and responses of the individuals within a culture.

In the foregoing experiment the bacteria were left in the same medium in which they had developed, and to which the salt had been added. It was conceivable that fresh saline broth might have a different effect on the viability of the cells in NaCl-containing media, even if they could not divide in it.

To test this, bacteria were acclimatized as in the previous experiment, and left in the same medium for 18 hours at 35°C., when 99 parts of fresh broth containing 7 per cent NaCl and 0.5 per cent buffer were inoculated with one part of the culture, and the resulting suspension was kept constantly stirred in a water bath at 0°C. for 6 days. Samples were taken out at intervals and dilution counts made in both 7 per cent and 8 per cent NaCl broth. The results are shown in Fig. 5 B.

While the low temperature prevents development in the freshly prepared saline medium, it has no appreciable effect on the ability of the cells to

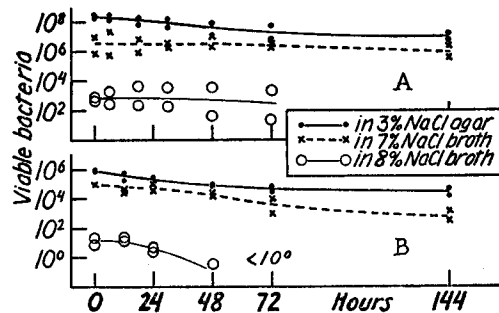


FIG. 5. Storage of acclimatized bacteria in saline broth.

A. Bacteria left at 35°C. in the medium in which they had developed, but with 7 per cent NaCl.

B. Bacteria placed in freshly prepared 7 per cent NaCl broth and kept at 0°C.

multiply in saline broth at a more favorable temperature. The curves are quite comparable to those in Fig. 5A if it is remembered that the initial points in Fig. 5B correspond to those at 18 hours in the upper graph, and that the cultures have been diluted 100-fold.

The only significant difference between the curves in Fig. 5A and 5B is that the viable counts in 8 per cent NaCl broth in the last experiment seem to diminish more rapidly than in the previous one. This difference may be ascribed to the combined inhibitory effects of high salinity and low temperature on the adaptive process.

2. *Studies on Successive Transfers in Saline Broth*

It had thus been ascertained that a prolonged exposure of acclimatized bacteria to a saline environment in which no detectable development takes place does not result in a further increase in the salt-viable counts beyond the maximum reached during the initial acclimatization process. The development of *E. coli* in strongly saline media could now be investigated with a view to establishing whether or not the cells produced in the presence of salt would show different characteristics with respect to their adaptive powers from those previously studied.

Bacteria from a 24 hour fresh-water culture were exposed to increasing salt concentrations in seven 1 per cent steps of 1 hour duration each, and left in 7 per cent saline medium for 18 hours. Transfers from this adapted culture were made in yeast extract with 7 per cent NaCl, and successive subcultures in this medium started every 48 hours. During the development of the first, third, and fifth subcultures viable counts were made in 7 per cent and 8 per cent NaCl broth, as well as on 3 per cent NaCl agar plates. Counts on the second and fourth subcultures were made only 48 hours after inoculation. The composite results of two such experiments are plotted in Fig. 6A, B, and C, in which the curves represent the counts obtained in the first, third, and fifth subcultures respectively.

From Fig. 6A it will be seen that the number of bacteria viable in 7 per cent NaCl broth, although initially only a fraction of the total number of living cells, soon began to increase, this increase becoming logarithmic with time, although the total number of living organisms did not increase until the number of cells viable in 7 per cent NaCl medium had approached it. Thereafter the two curves coincided for a period of time extending slightly beyond the logarithmic phase.

Consequently only those cells originally viable in 7 per cent NaCl broth are capable of reproducing in this medium, while the others do not divide at all. The logarithmic order of growth indicates that all cells produced in saline medium can continue to reproduce in it. This is supported also by

the close agreement between the counts on 3 per cent and 7 per cent NaCl media in the subcultures. In addition, no discrepancy was observed between the viable and the direct microscopic counts during the logarithmic period in any of these experiments.

The 7 per cent NaCl-viable count falls below the viable count on 3 per cent NaCl plates with senescence of the culture. This suggests that even bacteria, grown in a strongly saline medium lose with age, their ability to propagate in such a medium although they are still capable of reproducing under more favorable circumstances.

An examination of the curves for the viable counts in 8 per cent NaCl broth in Fig. 6 A, B, and C reveals that they roughly parallel those for the counts in 7 per cent NaCl media. Consequently, the ratio of the number of organisms viable in 8 per cent to that in 7 per cent NaCl remains almost constant throughout. This warrants the conclusion that repeated transfers in saline broth do not cause any further acclimatization of the organisms to more saline environments.

The ratio of 8 per cent NaCl-viable bacteria to the total number of bacteria present is higher in these experiments than it was after the "non-selective acclimatization" of cultures grown in NaCl-free media (*Cf.*, *e.g.*, Fig. 2). This might seem to imply a further acclimatization to higher salt concentrations, but a careful analysis of all the data obtained would rather support a different conclusion. The selective propagation of those cells capable of reproducing in 7 per cent NaCl media, combined with the ability of a constant fraction of such cells to develop in higher salt concentrations, would obviously yield the observed results. In view of the demonstration that the ratio between the numbers of bacteria viable in 7 per cent and in 8 per cent NaCl broth is practically constant, and that in 7 per cent NaCl broth no increase in 3 per cent NaCl-viable bacteria was found prior to an increase in 7 per cent NaCl-viable cells up to the initial number of the

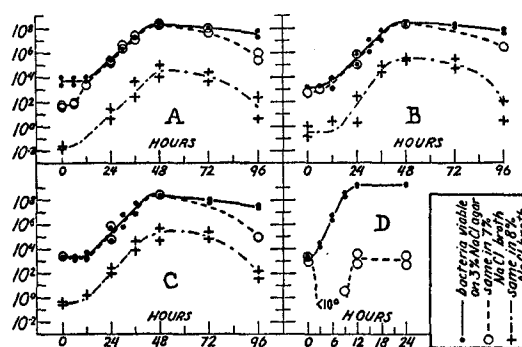


FIG. 6. Development and adaptability of *E. coli* in saline and salt-free broth.

- A. 1st subculture in 7 per cent NaCl broth.
- B. 3rd subculture in 7 per cent NaCl broth.
- C. 5th subculture in 7 per cent NaCl broth.
- D. Development in salt-free broth after three transfers in 7 per cent NaCl medium.

former, the interpretation based on selection instead of individual acclimatization must be deemed more probably correct.

A number of investigators have obtained adaptation to unfavorable environments by culturing the organisms under conditions intermediate between the "normal" and the altered habitat. The results here reported indicate that the mechanism of such adaptation would consist of both individual acclimatization, which is independent of reproduction, and of selection by the environment of those cells endowed with a wider range of tolerance to normally unfavorable conditions.

Both the growth rate and the maximum crop are greatly diminished in the presence of high salt concentrations. This is clearly brought out in Table VIII, in which the minimum division times and the maximum crops

TABLE VIII
*Minimum Division Times and Maximum Crops in Fresh and Saline Media**

	Minimum division time	Maximum crop
	<i>min.</i>	
In salt-free medium.....	24	1.8×10^9
In 7 per cent NaCl broth:		
1st subculture.....	103	1.8×10^8
2nd ".....		1.6×10^8
3d ".....	104	1.7×10^8
4th ".....		1.9×10^8
5th ".....	103	1.9×10^8

* The figures represent means of two experiments in each case.

in saline and in salt-free cultures are compared. These findings agree with those of Eisenberg (37), Estor (38), Speakman, Gee, and Luck (39), and Vaas (16).

From the curves in Fig. 6A, B, and C, and particularly from Table VIII, it is clear that repeated transfers in the 7 per cent NaCl broth do not lead either to a faster growth of the bacteria or to a larger crop than can be obtained with the first saline culture. These observations further support the contention that such transfers do not yield bacteria with increasingly greater potentialities but simply a larger number of acclimatized cells by a process of selection.

The morphological characteristics of cultures in media without salt differed from those in concentrated saline broth. In non-aerated salt-water cultures the growth was stringy and mucoid; the organisms formed a sediment reminiscent of agglutinated bacteria. This was never observed in fresh-water cultures, where the medium became homogeneously turbid;

and even in old cultures, where precipitation had occurred, the deposit could readily be shaken up again to a uniform suspension. Similar differences, though less marked, could be observed with aerated cultures. Microscopic examination showed that in the NaCl-containing media the individual cells have a strong tendency to hang together; the bacteria are non-motile and form chains of appreciable length. During the logarithmic growth the organisms were distinctly longer in saline than in salt-free broth, but in the stationary phase the size of the bacteria was again reduced approximately to normalcy.

In very old NaCl-broth cultures the liquid became brown; such discoloration was never observed in fresh-water media.

3. Studies on the Behavior of Saline Broth Cultures upon Return to Fresh-Water Media

It has been shown that the manifestations of the individual acclimatization, induced by exposure of non-dividing cells to intermediate salt concentrations, rapidly disappeared when the cells were re-suspended in a salt-free environment.

Inasmuch as the experiments presented in the previous section have failed to demonstrate a detectable difference between cells thus acclimatized and new cells produced by frequent subcultures in concentrated NaCl media, it seemed probable that a reversibility of the adaptation process could also be proved for the latter.

After three transfers in 7 per cent NaCl broth, carried out as in the last experiment, a subculture was made in fresh salt-free medium. Viable counts on yeast agar as well as in 7 per cent NaCl broth were made during its development. The results of two such experiments are shown in Fig. 6D, while the mean minimum division rate and maximum crop under these conditions are presented in Table IX, together with those previously found in fresh-water and saline cultures.

From Fig. 6D it may be seen that there was hardly any lag phase and no death of bacteria following the transfer of saline-broth cultures to fresh-water medium. Yet, such phenomena might have been expected from the studies of Kluyver and Baars (13) and of Hoare (4). It must, however, be remembered that the potentialities of the organisms used by these investigators were apparently much more limited than those of *E. coli* and *B. megatherium* because after their cultures had been adapted to a new environment by a series of successive subcultures under progressively modified conditions the majority of the cells had become incapable of reproducing in the original one. Such limitations of potentialities might,

through continued selection, bring about a segregation of populations which are, in some physiological aspects, sufficiently different to suggest separate species.

It is also apparent from Fig. 6D and from Table IX that the division rate as well as the total crop immediately revert to their normal values for fresh-water broth. This definitely bars the possibility that the salt-tolerant cells of *E. coli* selected by the cultivation in a saline medium differ in their intrinsic reproductive powers from those incapable of dividing in the presence of high NaCl concentrations.

The viable count in 7 per cent NaCl broth, roughly equal to the total viable count at the beginning of the experiment, is seen to decrease so rapidly that 2 hours after inoculation it can no longer be computed. At the end of the logarithmic phase the number of bacteria viable in 7 per

TABLE IX
*Comparison between Growth Rates and Crops in Salt-Free and Saline Media**

	Minimum division time	Maximum crop
	<i>min.</i>	
In salt-free medium.....	24	1.8×10^9
In 7 per cent NaCl broth.....	103-104	$1.6-1.9 \times 10^8$
In salt-free medium after 3 transfers in 7 per cent NaCl broth.....	25	1.9×10^9

* The figures represent means of two experiments in each case.

cent NaCl again rises to the approximate value of similar counts made with ordinary fresh-water cultures never exposed to salt. This agrees with the findings of Vaas (16) and proves that whatever may be the difference between individuals viable and non-viable in saline broth, it certainly is not an hereditary distinction, and that the adaptation here studied can not even be classed as a "*Dauermodifikation*" as defined by Jollos (1).

Thus, the ability to reproduce in an unfavorably saline medium, acquired through the formation of new cells in such a medium, is lost as completely as that acquired by the exposure of non-dividing cells to intermediate salt concentrations after the return of the acclimatized bacteria to a salt-free environment.

V

GENERAL DISCUSSION AND CONCLUSIONS

The present treatise was meant as an exploration of but one of the many phases of the problem of adaptation of microorganisms to their

environment. It is apparent that many factors are involved in the adaptation of *E. coli* to saline media, each of which must be investigated in detail before it can be hoped that an understanding of the reactions involved will be reached.

The standard technique as developed here makes it possible to use the changes in the ability of bacteria to reproduce under altered conditions as a criterion of their "adaptive power" with nearly the same accuracy as can be attained in ordinary determinations of "death points" or "death times."

The experiments presented strongly support, if not conclusively prove, Vaas' hypothesis of a "fluctuating variation" among individuals in a pure culture with respect to their ability to develop in a modified environment. That the limits of such variability are dependent on the *milieu* has been demonstrated more clearly than in his own experiments, for it has been shown that the range of tolerance can be extended without any reproduction or selection occurring in the cultures. This individual acclimatization has been demonstrated to be reversible and influenced by temperature. That it does not merely involve the recovery of the bacteria from osmotic effects produced by the salt, as proposed by Chatton and Tellier (5) in explanation of the behavior of protozoa, seems likely from the observation that subjection of the organisms to a very low intermediate NaCl concentration may widen the range of variation to its maximal limits.

The developmental phase of the culture influences the ability of the bacteria to grow in saline media in much the same manner as it does the resistance to toxic agents in general, suggesting a close relationship between adaptability and resistance.

Some investigators have emphasized the applicability of the law of mass action in conjunction with due consideration of the size of bacteria to studies of death rates of bacteria, concluding that all the individuals had the same resistance. (For a detailed discussion of these problems see Rahn (40, 41), Holwerda (42).) The experiments described here provide ample evidence of the actual occurrence of variation among the individuals with respect to their adaptive ability; hence the possibility that variation within a population would influence its statistical "death rate" may not be disregarded.

The selection by the medium of individuals possessing a wider range of potentialities has also been demonstrated in the present investigations. That the displacement of the "limits of variability" has no hereditary basis or consequence must be particularly emphasized.

By the analysis of viable counts and growth curves it has thus been

possible to separate the processes involved in the adaptation of *E. coli* to a new (saline) environment into two components, namely: acclimatization which apparently does not involve reproduction, and selection of individuals with the greatest potentialities.

The methods outlined in the present work for separating the two factors are perhaps not the only available nor the simplest means of attacking the problem, but the results seem to justify the expectation that they may be used with equal success for the study of a variety of related problems.

I wish to express my sincere gratitude to Dr. C. B. van Niel, whose valuable advice I have sought on numerous occasions, and whose unfailing enthusiasm has been a source of encouragement and stimulation.

SUMMARY

1. It has been shown that a fairly constant fraction of the total number of bacteria in a fresh-water culture of *E. coli* can reproduce on direct transfer to a saline medium with a definite NaCl concentration, as judged from the viable count determinations in such a medium.

2. The absolute value of this fraction depends on a number of factors other than the salt content of the test medium, such as the hydrogen ion and yeast autolysate concentrations, aeration, and the physiological condition of the bacteria.

3. A method for testing the degree and rate of adaptation of the bacteria to saline environment, depending on the analysis of changes in the value of the salt-viable fraction, was developed.

4. Maximum adaptability to saline environments was found during the early stationary phase of NaCl-free cultures. Low adaptability accompanied the logarithmic phase and the senescence of the cultures.

5. The limits of variation could be extended by treatment of non-dividing cells with gradually increasing concentrations of salt or by subjecting them to a single intermediate NaCl concentration. This acclimatization was independent of reproduction. The number of bacteria becoming capable of reproducing in a hitherto unfavorable environment increased with the period of exposure to intermediate salt concentrations until a maximum value was reached.

6. This maximum value was shown to depend on the salinity of the test medium, the age of the bacterial culture, and the method of preliminary treatment. "Optimal acclimatization" could be effected by subjecting the organisms to a single fairly low intermediate NaCl concentration.

7. The rate of the individual acclimatization process was shown to be greater at higher than at lower temperatures.

8. Acclimatized bacteria rapidly lost their increased ability to reproduce in saline media upon return to a salt-free environment, although no reproduction of the cells could be detected. This was interpreted as an indication that the processes involved are readily reversible.

9. Studies on the reproduction of *E. coli* in strongly saline broth indicated that only those cells originally acclimatized to the salt concentration of the medium could divide. All cells produced in such a medium could continue to reproduce. The propagation in the altered medium was not accompanied by any further acclimatization throughout five subcultures.

10. Both the division rate and the maximum crop of cultures in saline broth were considerably lower than of those in a fresh-water medium. No change in either occurred throughout five successive subcultures. The morphology of the organisms was also altered by the presence of salt.

11. The division rate, maximum crop, morphology, and adaptive power returned immediately to normal on re-transfer of bacteria grown in an NaCl-containing medium to "salt-free" broth.

12. The entire adaptive response of the bacteria to a considerable increase in the salinity of the environment could thus be separated into two components: an acclimatization, independent of reproduction, and a selection of those cells with the widest range of potentialities.

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