SALT DESIDERATUM OF VIBRIO COSTICOLUS, AN OBLIGATE HALOPHILIC BACTERIUM

I. Ionic Replacement of Sodium Chloride Requirement

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Halophilism has been of considerable interest since the report of Farlow (1880) on the reddening of salted fish. Any industry using either a salt packing process or a salt brine must be concerned with the control of halophiles. Considerable work has been done, therefore, with the isolation, identification, and control of these organisms. Stuart, Frey, and James (1933), Gibbons (1936), Lefevre and Round (1919), Harrison and Kennedy (1922), Kellerman (1914), and others have reviewed the literature concerned with this work. In contrast to this work very few studies have been carried out on the effect of environmental conditions established by salt on these organisms. This is due, in part, to the problems encountered in cultivation since almost all obligate halophiles grow slowly and best on solid media.

The purpose of this paper is to present results of an investigation on the salt requirement of an obligate halophilic bacterium, *Vibrio costicolus*,[1] with particular reference to the replacement of its sodium chloride requirement with a variety of cations and anions.

MATERIALS AND METHODS

Culture and inoculum. Stock cultures of *V. costicolus* were carried on slants composed of 0.5 per cent yeast extract, 1 per cent trypticase, 1 M sodium chloride, and 2 per cent agar. *V. costicolus*, first isolated and described by Smith (1938), is a gram negative curved rod with a single terminal flagellum. No spores are formed. No variation in size or shape in different concentrations of sodium chloride was noted. The formation of a pellicle was pronounced in broth cultures after 24 hours. With the exception of maltose and galactose, which were fermented, the biochemical reactions resembled those given by Smith. The inoculum used in all experiments was one drop of a twice-washed 24 hour culture grown in broth composed of 1 M sodium chloride and 1 per cent trypticase.

Basal medium. Preliminary studies revealed that 1 per cent trypticase (Baltimore Biological Laboratories) in water, adjusted to pH 7.2, gave excellent growth response when the necessary amount of sodium chloride was added. The trypticase broth was prepared in large quantities in 10 per cent concentration and dispensed in 100 ml lots into rubber stoppered bottles, sterilized by autoclaving, and then refrigerated until used. It was prepared in quantity to eliminate variation in the nutritional properties of the medium.

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Preparation of salt solutions. A total of 12 salts (Baker's analyzed grade) were used. No effort was made to purify them further since the experiments were designed to study the effect of large concentrations of ions rather than trace amounts. Concentrated stock solutions of the salts were prepared, adjusted to pH 7.2, and working solutions for each salt were made from the stock solutions. Working solutions with concentrations 0 to 4 M in 0.2 M steps were prepared of sodium chloride, sodium bromide, sodium iodide, sodium nitrate, lithium chloride, and magnesium chloride. Concentrations from 0 to 2.6 M for sodium sulfate, to 1.8 M for sodium molybdate, to 1.6 M for sodium phosphate (mono- and dihydrogen mixture), to 1.0 M for sodium fluoride, to 2.4 M for potassium nitrate, and to 3.6 M for potassium chloride were prepared in 0.2 M steps.

Experimental procedure. Eight ml of the working solutions for a particular salt were added to a series of tubes. Then the tubes were capped and sterilized by autoclaving. After cooling, 1 ml of sterile 10 per cent trypticase broth and 1 ml of sterile water were added aseptically to each tube. The 1 ml of water was replaced by 1 ml of certain salt solutions in subsequent experiments. The salt solution, trypticase, and water were mixed by gentle shaking and allowed to stand at least 4 hours before inoculation. This was done to be certain that no precipitation of any material occurred. The tubes then were inoculated and incubated 48 hours at 32 C. Immediately following the incubation interval the cell density in the tubes was determined by a Klett-Summerson photoelectric colorimeter using a blue (400 to 465 mμ) filter. The readings obtained are expressed as per cent light transmittance and plotted against the concentration of salt used.

In measuring the response of *V. costicolus* to sodium chloride, 20 tubes containing the various concentrations of sodium chloride described above were prepared. Another series of experiments was made to determine the specificity of the sodium chloride requirement. The substitution salts and concentrations used in these experiments also are described above. These particular salts were selected because of their common ion relationship, high solubility, and formation of colorless solutions. The substitution experiments were performed in the same manner as the sodium chloride experiments. The cation and anion substitution experiments were then repeated but with the addition of 0.2 M sodium chloride to each concentration of all salts and 0.4 M sodium chloride to each concentration of a second set.

RESULTS

Response of V. costicolus to sodium chloride. It can be seen in figure 1-D that the response of the organism began at 0.4 M and increased until it reached a peak at 1.2 to 1.4 M and then declined as the concentration of the salt continued to increase until no response was obtained at 3.6 to 3.8 M. This was always the case in 30 independent trials. Even after one week of incubation the organism failed to respond in concentrations of sodium chloride below 0.4 M or above 3.8 M.

Response to cation and anion substitution. The response of *V. costicolus* to anion substitution is shown in figure 1-A. The sulfate ion has been substituted for the added chloride ion. The response is of a similar magnitude to that obtained with sodium chloride but is not within the same limits of concentration. The entire curve has shifted to the left with the maximum response being obtained at 0.6 M. When 0.2 M sodium chloride was added to similar concentrations of sodium sulfate, a curve was obtained, as shown in figure 1-B, with the shift to the left even more pronounced but with little change in the magnitude of response. The addition of 0.4 M sodium chloride had little effect on the response curve other than to accentuate the shift to the left. Similar results were obtained with other anion substituting salts, such as sodium molybdate, sodium phosphate, and sodium bromide, although the magnitude of response and limiting concentration varied with the individual salt. No response was obtained with sodium fluoride, sodium iodide, potassium nitrate, and sodium nitrate alone; when 0.2 M sodium

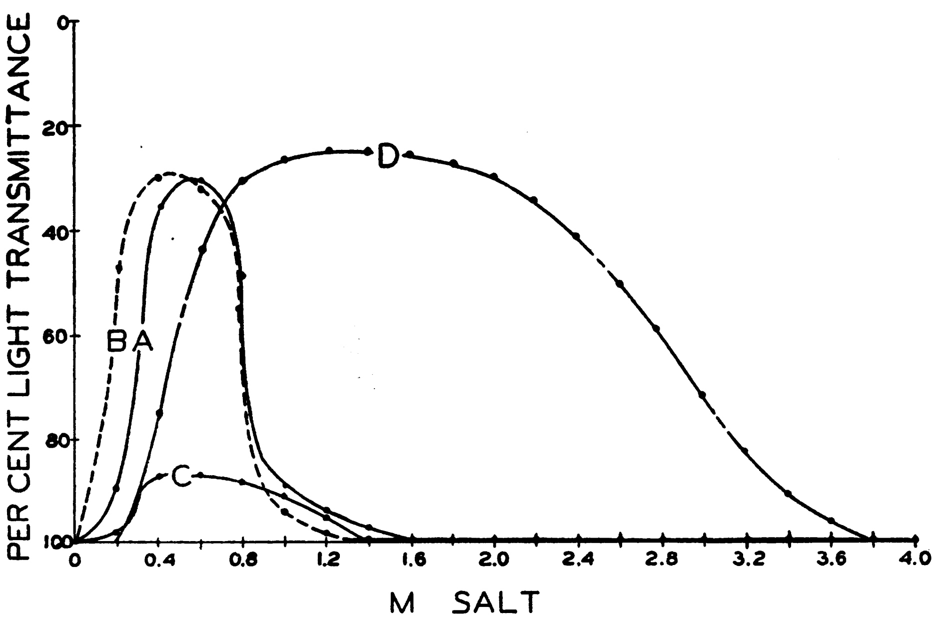

Figure 1. Response curves of *Vibrio costicolus* to varying concentrations of: (A) sodium sulfate; (B) sodium sulfate plus 0.2 M sodium chloride; (C) magnesium chloride; and (D) sodium chloride.

chloride was added to each concentration, sodium iodide, potassium nitrate, and sodium nitrate gave response curves similar to those obtained previously with other salts. Of all the salts used only sodium fluoride failed to allow growth even though concentrations of sodium chloride as great as 3.8 M were added. The lowest concentration of sodium fluoride used was 0.2 M, and it must be remembered that this is rather concentrated for a salt as toxic as sodium fluoride.

The response of *V. costicolus* to cation substitution is shown in figure 1-C. This figure illustrates the response obtained when the magnesium ion is substituted for the sodium ion. Similar curves were obtained with other cation substituting salts, such as potassium chloride and lithium chloride, although the magnitude of response varied slightly with the particular salt. When 0.2 M and 0.4 M sodium chloride were added to all concentrations of these salts, the magnitude of response was greatly increased; curves obtained were almost identical to the curve obtained with sodium sulfate and added sodium chloride (figure 1-B).

DISCUSSION

From the results presented it is evident that the sodium chloride requirement of *V. costicolus* is not specific. Other laboratories are investigating this phenomenon also (Gibbons, 1952). It should be clearly understood, however, that trace amounts of sodium and chloride ions may be required by the organism.

With salts in which anion substitution was made sulfate, molybdate, and bromide were found to satisfy the ionic requirement almost as well as chloride. When cation substitutions were made, magnesium, potassium, and lithium only partially satisfied the requirement, and the magnitude of response was on a much lower level than with sodium. In general, retention of the sodium ion gave better response than retention of the chloride ion. Either the sodium ion is more stimulatory than the chloride ion or the substituted cations are more inhibitory than the substituted anions.

It seems from the results obtained that the fluoride, nitrate, and iodide ions do not satisfy the organism's physiological requirements and may even be toxic. Fluoride is definitely toxic, for no response was obtained with any concentration or combination of salts. Eisenberg (1918), when working with nonhalophiles, found these anions to be more toxic than sulfate, chloride, and bromide in the presence of sodium. A list of the anions in descending order of response, chloride, sulfate, molybdate, bromide, phosphate, nitrate, iodide, and fluoride, shows the same general pattern as reported by Eisenberg (1918), Holm and Sherman (1921), and others of increasing toxicity on nonhalophiles. The influence of various anions in the presence of sodium (Eisenberg, 1918) and the influence of various cations in the presence of chloride (Hotchkiss, 1923) have been studied thoroughly using nonhalophiles. It may be of interest to note that if small amounts of sodium chloride are added, a response to sodium or potassium nitrate and sodium iodide, but not to sodium fluoride, is obtained. This would seem to indicate that the chloride ion is of some importance to this organism. In view of the responses obtained with other salts and combinations of salts it seems more likely that the chloride ion may be antagonistic to the nitrate and iodide ions.

There is some evidence that osmotic pressure may be of great importance to this organism. Lefevre and Round (1919) suggested that osmotic pressure might be important to halophiles, and they felt that this might be proven if other salts could replace the sodium chloride requirement. There is more in the data presented here that suggests an osmotic pressure explanation for halophilism than just a substitution of salts for sodium chloride. Note in figure 1-D the maximum response was obtained at 1.2 M sodium chloride. Multiplying 1.2 by 2, since sodium chloride is a 2 ion salt, a product of 2.4 representing the ions present is obtained. In figure 1-A and 1-C the maximum response is at 0.6 M. These curves represent the response to sodium sulfate and magnesium chloride. Since they are 3 ion salts, the concentration of 0.6 M is multiplied by 3 and a product of 1.8 representing the ions present is obtained. This is very close to the 2.4 obtained for sodium chloride if the difference in ionic activities and interionic relationships is kept in mind. The maximum response of the organism to sodium sulfate plus 0.2 M sodium chloride is represented in figure 1-B as being at 0.4 M. Multiplying 0.4 by 3 gives 1.2 and adding 0.4, which represents the ions of the added 0.2 M sodium chloride, gives a total of 1.6. This is also very close to the 1.8 for magnesium chloride and for sodium sulfate and to the 2.4 for sodium chloride. The similarity in the concentration of ions necessary to give maximum response seems to indicate that the number of particles present in the medium is of great importance to the organism. Osmotic pressure is one of several properties which are influenced by number, rather than kind, of particles. This particle influence is being investigated more thoroughly at the present time.

SUMMARY

By using concentrations of sodium chloride from 0 to 4 M in 0.2 M steps it has been possible to demonstrate that *Vibrio costicolus*, an obligate halophile, responds to various concentrations of sodium chloride in a regular and measurable manner.

It has also been demonstrated that the requirement for sodium chloride is not specific, for other salts (sodium sulfate, sodium molybdate, sodium phosphate, sodium bromide, magnesium chloride, potassium chloride, and lithium chloride) were able to satisfy completely the requirement of the organism for added sodium chloride. Cation and anion substitution studies demonstrated that both the cation (sodium) and the anion (chloride) of the added sodium chloride could be completely replaced by other ions.

The results obtained also suggest that the cation (sodium) has more of a stimulatory effect upon the response of the organism than does the anion (chloride).

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