A MECHANISM OF HALOPHILISM IN MICROCOCCUS HALODENITRIFICANS[1]

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Received for publication December 23, 1951

Halophilic bacteria are characterized by their ability to grow in media containing concentrations of sodium chloride that usually completely inhibit the multiplication of nonhalophilic species. Data to provide a complete explanation of this resistance to high concentrations of salt are not available, though several authors have implied that the protoplasm or certain enzymes of halophilic cells differ from their nonhalophile counterparts in a manner which confers specific resistance to inactivation by salt.

Resistance to salt also could be conferred if the halophilic cells were able to limit the penetration of salt into the cell, either by some inherent physical characteristic of the external membrane of the cell or by some active mechanism which could prevent diffusion of an easily diffusible solute such as sodium chloride.

Since salt is thought to diffuse through a hydrophilic phase of the "normal" plasma membrane, it might be argued that diffusion of salt might be impeded if the halophilic cell surface were predominantly hydrophobic (e.g., lipid).

Mechanisms capable of controlling permeability have frequently been postulated (Krogh, 1946; Monné, 1948; Steinbach, 1951), but no specific mechanism has been established.

Accordingly, the three concepts previously indicated have been explored experimentally. These concepts are: (1) that the proteins of the cell matrix of halophilic bacteria are specifically resistant to inactivation by salt; (2) that the cells of halophilic bacteria are coated with a lipid material which may impede the diffusion of salt; and (3) that the diffusion of salt into the cell is restricted by the expenditure of energy derived from some physiological process in the cell.

METHODS

A halophilic coccus, *Micrococcus halodenitrificans* (Robinson and Gibbons, 1952), was used as the test organism. The activity of lactic acid dehydrogenase in washed cell suspensions and of nitritase in washed cell suspensions and cell-free preparations was determined by the methods described by Robinson (1952).

Cell-free preparations were prepared by the method of Kalnitsky, Utter,

[1] This work, submitted in partial fulfillment of the Ph.D. requirements, McGill University, was aided by an N. R. C. scholarship held by the senior author. Issued as N. R. C. no. 2672.

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and Werkman (1945). Cell paste, powdered glass, and 3.28 per cent sodium chloride solution were mixed in the proportion of 1:3:1 by weight and passed between two rotating, chilled, ground glass cones.

Electrophoretic mobilities of the test organism were determined in the Abramson modification of the Northrup-Kunitz microelectrophoretic cell employing the standard method of Moyer (1936). The applied voltage was maintained at a constant level by means of a voltage regulated direct current power supply (Robinson, 1950). Stock washed cell suspensions of the test organism were prepared from 18 to 24 hour cultures in 3.28 per cent sodium chloride solution. The cells were washed twice the same morning that electrophoretic mobilities were measured and resuspended in phosphate buffer (0.01 M) or in buffer containing 0.6 M sodium chloride. To minimize the possibility of chemical contamination of the electrophoretic cell and of change in mobility of the bacteria with time, mobilities of a given cell suspension were determined in random order. All results represent the average mobilities of 20 different cells with replications on three different days using different cell suspensions.

RESULTS

Protein solubility. Many substances, including the common proteins, are "salted out" by solutions of salts. This well known phenomenon proceeds gradually and regularly with increasing salt concentrations and can be related to the ionic strength of the solution (Ingram, 1947).

Halophilic bacteria grow and metabolize in nutrient media containing concentrations of salt which would induce at least partial salting out of the common proteins. The extraordinarily high specific gravity of the cells of a halophilic purple *Spirillum* suggested to Baas-Becking (1928) that the protoplasmic constituents of this and similar organisms were not highly hydrated and, therefore, were abnormally resistant to salt inactivation. Baumgartner (1937) appeared to favor the idea that the proteins of halophilic bacteria were characterized by a pronounced solubility in concentrated salt solution.

Ingram (1947) noted that the resistance to inactivation by salt of a lactic acid dehydrogenase isolated from *Escherichia coli* increased progressively as the enzyme was purified. He suggests that although the active groups of the enzymes may be of moderate molecular size they may be associated in the cell with large aggregates and thus be less soluble in salt solutions. By analogy, he suggests that the enzymes of the halophiles are not linked with these large aggregates. Such enzymes would be expected to exhibit maximum activity at the salt concentration in which they are most soluble.

In this study the activity of nitritase, the enzyme which reduces nitrite to nitrogen, has been used as a measure of the effect of salt on an intracellular protoplasmic constituent. Preliminary tests indicated that the production of nitrogen was a linear function of the concentration of cells with maximum nitritase activity of washed cell suspensions at 2.2 per cent sodium chloride (figure 3, and Robinson, 1952). In cell-free preparations the activity of the enzyme was reduced considerably (table 1), but the results indicated that maximum enzyme

activity occurred at a lower salt concentration than in the intact cells. The average nitritase activity of three different cell-free preparations determined in duplicate is presented in figure 1. Enzyme activity was maximal in 0.9 per cent sodium chloride. Although some viable cells remained (table 1) in the cell paste, it is unlikely that they contributed significantly to the production of N_2 considering that 10^{10} cells produced only 66.5 μL of nitrogen in 2.2 per cent sodium chloride solutions.

In cell-free preparations nitritase was partially inactivated, possibly by the salting out reaction, at the salt concentration in which *M. halodenitrificans* grew optimally (4.4 per cent, Robinson and Gibbons, 1952). This result suggests that the salt content of the cell may be less than that of the medium, and this interpretation is further borne out by the fact that although the cells were ground with an equal volume of 3.28 per cent sodium chloride solution, the salt content of cell-free preparations was only 2.2 to 2.4 per cent. Unpublished results obtained by

TABLE 1

The quantitative recovery of nitritase

(μL N_2 per 30 minutes per 2.0 mg test material)

PER CENT NaCl IN REACTION VESSELS	NITRITASE ACTIVITY		
	Powdered glass-bacterial paste mixture		
	Before grinding*	After grinding*	Cell-free preparation*
0.55	24.2	14.9	8.5
1.1	54.4	23.4	11.0
2.2	66.5	31.2	8.6

* Log viable cell count 10.4, 8.9, 4.4, respectively.

one of us (N. E. G.) indicate that the salt concentration in cells of halophiles is considerably less than that of the medium in which they are growing (Ingram, 1947).

It must be concluded that at least one constituent of the cell contents of this organism is adversely affected by low concentrations of salt and is similar in this respect to the cell constituents of nonhalophiles. In this instance, therefore, halophilism cannot be attributed to the presence of enzymes that resist relatively high salt concentrations.

Salt impermeable layer. If, as indicated in the previous section, some of the proteins of halophiles are sensitive to salt and the salt concentration within their cells is less than that of the medium, it must be concluded that salt is prevented from entering the cells.

Knaysi (1944) and Monné (1948) have shown that the cytoplasmic membrane of many bacteria is lipoidal in composition. Weatherby (1949) observed that artificial membranes prepared to contain lecithin, cephalin, or mixtures of the two, were highly impermeable to ions of organic and inorganic electrolytes but were quite permeable to nonionized molecules. Hence, if it is assumed that salts normally penetrate the plasma membrane in the ionized state, it would follow

that bacterial membranes containing more than the usual amounts of lipids would retard diffusion of sodium and chloride ions into the cell.

Dyar and Ordal (1946) have demonstrated differences in the lipid content of the bacterial membranes using the electrophoretic technique of Moyer (1936). Dyar (1948) offered experimental evidence that lipid droplets, or bacterial cells with lipid materials at the surface, possess greater negative charges (and hence increased electrophoretic mobility) "when suspended in neutral or slightly alkaline buffer solutions containing anionic surface active agents than when in buffer alone." On the other hand, various protein and carbohydrate materials did not behave in this manner. According to Dyar, this difference in behavior would permit estimation of the proportion of the cell area covered with lipid. Her technique

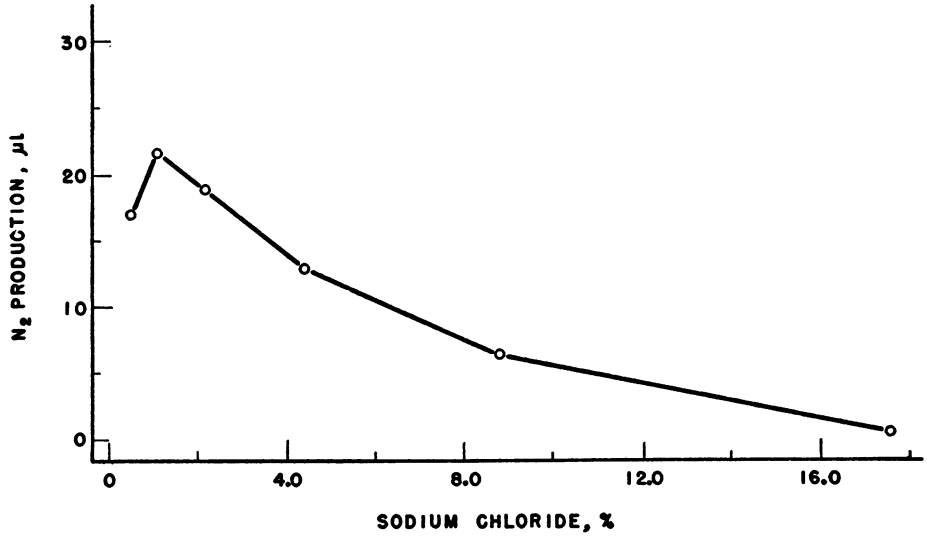

Figure 1. The response of cell-free nitritase preparations of *Micrococcus halodenitrificans* to sodium chloride.

was used, therefore, to investigate the possible presence of lipid on the cell surfaces of *M. halodenitrificans* (figure 2).

The mobility of cells suspended in salt-free buffer was quite high and was not affected by the addition of the anionic detergent, dioctyl sodium sulfo-succinate ("aerosol OT"). When the cationic detergent, cetyl pyridinium chloride, was added, the charge diminished and the polarity finally reversed as the concentration of detergent increased. Mobility was very sluggish in salt-phosphate buffer, the addition of cetyl pyridinium chloride decreasing the mobility slightly. Aerosol, however, had little effect. Much of the detergent was "salted out" at a molar concentration of 10^{-3}, and the effect of these higher concentrations could not be evaluated. According to the argument advanced by Dyar and Ordal, these results indicate that the surface of *M. halodenitrificans* is nonlipoidal in nature.

Lipoidal material could not be detected at the surface of cells allowed to stand

overnight in salt-free or salt-containing buffer and stained by the method of Burdon (1946). Cells suspended in salt phosphate buffer stained readily, and fat globules could be detected within the cells; cells suspended in salt-free buffer appeared as typical ghost cells, and no globules could be detected.

Although the validity of the interpretation of the electrophoretic data may be open to question, there seems to be little evidence that there is a high surface concentration of lipoidal material that would aid in the exclusion of salt from the cell.

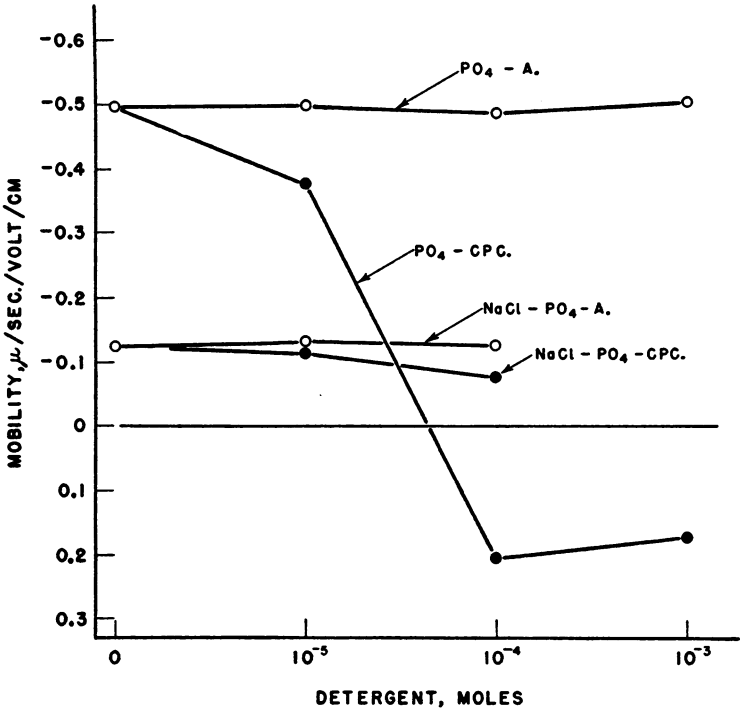

Figure 2. The electrophoretic mobility of cells of *Micrococcus halodenitrificans* suspended in sodium chloride-phosphate and in phosphate buffers in the presence of "aerosol OT" (A) and cetyl pyridinium chloride (CPC).

Salt excluded by the expenditure of energy. Halophilism may also be explained by the presence of some intracellular energy mechanism that regulates salt diffusion into and water diffusion out of the halophilic cell. There is also the possibility that the cell membrane of halophiles is characteristically impermeable to sodium and chloride ions and that an energy mechanism maintains the balance between internal and external water concentrations. However, as pointed out before, the cell appears to contain some salt so that regulation of ions rather than of water seems the more likely explanation.

Energy mechanisms have been associated with a number of cases of active diffusion. Patterson and Stetton (1949), Davies and Terner (1949), and Davies

and Ogston (1950) studied the mechanism whereby the parietal cells of the stomach secrete hydrochloric acid against a concentration gradient and are of the opinion that the energy necessary to secrete the acid was derived from carbohydrate oxidation.

Much work has been done on the sodium and potassium relations in the red blood cell, and it now seems quite clear that the elimination of sodium is linked with the energy released by glycolysis. More recent work (Weller and Taylor,

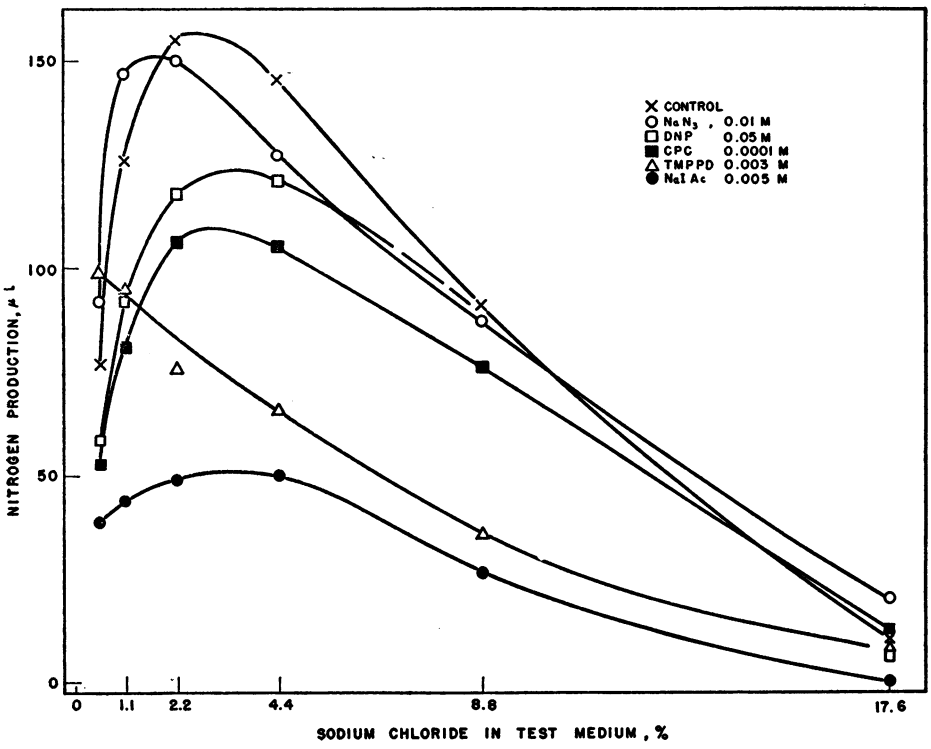

Figure 3. Salt response of nitritase of intact cells of *Micrococcus halodenitrificans* in the presence of dinitrophenol (DNP), sodium azide, cetyl pyridinium chloride (CPC), sodium iodoacetate, and phenylenediamine (TMPPD). Activity expressed as μL per 2 mg cells per 30 minutes.

1950; Lindvig, Greig, and Peterson, 1951) suggests that cholinesterase may also play a part. Scott, Jacobson, and Rice (1951) have shown that the internal concentration of sodium increases and potassium decreases when the glycolytic system of *Saccharomyces cerevisiae* is poisoned with iodoacetic acid or sodium fluoride.

If an energy mechanism is responsible for maintaining the difference between internal and external salt concentrations in halophiles, inactivation of this mechanism should allow equilibrium to be established and the nitritase activity of intact cell suspensions should decrease as a direct result of sodium chloride in-

activation. In these studies dinitrophenol, sodium azide, and cetyl pyridinium chloride were used as potential inhibitors of electron and phosphate bond transfer mechanisms. Tetramethyl-*p*-phenylenediamine was used as an inhibitor of coenzyme 1 and 2 energy linked mechanisms (Patterson and Stetton, 1949).

Preliminary studies indicated that, in the presence of 2.2 per cent sodium chloride, 0.01 M azide did not reduce nitritase activity, 0.05 M dinitrophenol reduced nitritase activity about one-quarter, 0.0001 M cetyl pyridinium chloride about one-third, 0.003 M tetramethyl-*p*-phenylenediamine about one-half, and 0.005 M iodoacetate about two-thirds. These concentrations of inhibitors and noninhibitors were added to intact cell suspensions of organisms grown in media containing 3.5 per cent salt and suspended in 0.55, 1.1, 2.2, 4.4, 8.8, and 17.6 per cent sodium chloride solutions. Each test was replicated three to five times on different days. The results were examined statistically[4] (Section 27, 28, Fisher, 1946), and the trends are plotted on figure 3.

Dinitrophenol, cetyl pyridinium chloride, and iodoacetate inhibited nitritase activity but did not affect the shape of the curves. Azide did not inhibit nitritase activity appreciably but shifted the maximum to the left to a significant extent suggesting a greater permeability of the cell to sodium chloride. Tetramethyl-*p*-phenylenediamine not only reduced nitritase activity but also produced a marked shift in the optimum salt concentration for nitritase activity. Iodoacetate produced a marked reduction in nitritase activity, but when lower cell to substrate ratios were used a curve resembling that of tetramethyl-*p*-phenylenediamine in shape was obtained. However, at the low cell concentration, nitritase activity was very low, and it is difficult to tell whether iodoacetate inhibited an energy mechanism or nitritase itself.

From the foregoing data it seems that while nitritase is incompletely inhibited by azide and tetramethyl-*p*-phenylenediamine the energy mechanism responsible for maintaining the salt or water differential between this halophilic organism and its environment is more completely inhibited by the same compounds. This interpretation suggests that a coenzyme 1 or 2 linked mechanism possibly coupled with a cytochrome-cytochrome oxidase system may be involved.

DISCUSSION

The observation that cell-free nitritase is sensitive to salt indicates that the concentration of salt within the cells of *M. halodenitrificans* is lower than the salt concentration of the medium and renders inapplicable the theory of halophilism based on resistance of protein constituents to "salting out". From results presented, it would also appear that the impermeability of the cell membrane to salt is not due to a lipoidal barrier at the cell surface. In the presence of tetramethyl-*p*-phenylenediamine, intracellular nitritase shows a reduction in activity with increasing salt concentrations similar in degree to that found for cell-free nitritase. It is, therefore, concluded that the salt concentration in the halophilic cell is normally kept below that of the environment by the expenditure of meta-

[4] The authors are indebted to Dr. J. W. Hopkins for the statistical analyses.

bolic energy. The inhibition by tetramethyl-*p*-phenylenediamine suggests a coenzyme 1 or 2 linked mechanism.

The expenditure of energy in this manner might explain the high salt tolerance of the organism used in this work but does not explain its salt dependence or its lack of growth in salt-free media. The expenditure of energy in controlling the passage of ions against an osmotic gradient, as visualized by Patterson and Stetton (1949), involves development of a high relative potential at the inner face of the "membrane" involved. If the postulated coenzyme 1 or 2 linked system is associated with maintenance of a comparable potential in the halophile cell, the establishment of such a potential in a cell present in a hypotonic medium would be expected to limit entrance of ionic metabolites from the external medium. Hence, the postulate could be extended to explain the absence of growth by the halophilic cultures when a high concentration of salt is not provided.

SUMMARY

In *Micrococcus halodenitrificans* denitrification proceeds optimally in cultures at a sodium chloride concentration of 2.2 per cent but in cell-free preparations at a concentration of 0.9 per cent. This indicates that the salt content of the cell is less than that of its environment and that at least one enzyme system is sensitive to "salting out". Electrophoretic data indicate that salt is not excluded from the cell by a lipoidal barrier. In resting cells denitrification proceeds optimally in the the presence of tetramethyl-*p*-phenylenediamine at a salt concentration of 0.55 per cent and in the presence of sodium azide between 1 and 2 per cent. It is concluded that these inhibitors interfere with an energy mechanism which maintains the concentration difference between the cell and its environment. The inhibition by tetramethyl-*p*-phenylenediamine suggests a coenzyme 1 or 2 linked mechanism.

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