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Mechanism of Optical Effects in Suspensions of a Marine Pseudomonad[1]

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When cells of a marine pseudomonad washed free of medium components with 0.05 M $MgSO_4$ were suspended in solutions containing 200-mM concentrations of various salts, there was an immediate increase in optical density (OD), followed by a slow decrease. The decrease following the initial increase, but not the increase itself, could be prevented by omitting K^+ from or by adding metabolic inhibitors to the suspending solution. With NaCl, the initial increase in OD rose to a maximum as the salt concentration was increased to 200 mM and then declined at 500 mM. There was a corresponding decrease in intracellular fluid volume to a minimum at 200-mM NaCl and then a rise. When the increased OD produced by NaCl was maintained, the internal Na^+ and Cl^- could be shown to have reached essentially the same concentration in the cells as in the medium. Thus, the OD changes could not have been due to osmotic effects. No evidence was obtained of a salt-induced aggregation of nuclear material. The OD of suspensions of isolated cell envelopes increased in response to increases in NaCl concentration in the absence but not in the presence of 0.05 M $MgSO_4$. The data was interpreted to indicate that the salt-induced increases in OD occurring in suspensions of the cells resulted from an interaction of salts with components of the cell envelope, causing contraction of the envelopes and shrinkage of the cells.

Turbidity changes occur in suspensions of gram-negative bacteria when the solute concentration of the suspending medium is increased by the addition of electrolytes or nonelectrolytes. The changes can often be separated into two phases. The first is an increase in turbidity which is complete within seconds. The second, which may or may not occur depending on the species (6), is a slow decrease in turbidity which follows the initial increase. These effects, which have been observed by a number of workers (1, 2, 6, 14-16), have been ascribed generally to an initial rapid decrease in size of the cells caused by the sudden increase in osmotic pressure in the suspending medium followed by a slow restoration of the cells to normal size as the solutes come into equilibrium across the osmotic barrier. Gram-positive bacteria ordinarily do not show such optical effects, although after incubation in phosphate buffer followed by washing in distilled water, the cells of a number of species of gram-positive bacteria became susceptible to optical changes comparable to those of gram-negative organisms (6, 9). Our interest in the mechanism of these optical effects was aroused when we observed that suspensions of a marine pseudomonad showed optical changes typical of those of other gram-negative bacteria upon the addition of NaCl to the suspending medium. Since previous studies had indicated that the cytoplasmic membrane of the marine pseudomonad presented no osmotic barrier to NaCl (19), it seemed unlikely that osmotic effects could account for the optical changes produced in suspensions of this organism. This paper provides evidence that the first-phage optical changes in suspensions of this organism are due to an interaction of salts with components of the cell envelope, whereas the second-phase changes require K^+ and are under metabolic control.

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MATERIALS AND METHODS

Culture. The organism used (ATCC 19855), referred to as marine pseudomonad B-16, was isolated origi-

nally from a marine clam and has been classified as a *Pseudomonas* species type IV. Studies on the nutrition and metabolism of this organism have been reported in some detail in previous communications, the most recent by Wong et al. (22).

Medium. The culture was maintained by monthly transfer on slants of a medium containing 0.8% Nutrient Broth (Difco), 0.5% yeast extract (Difco), and 1.5% agar in a salt solution consisting of 0.22 M NaCl, 0.026 M $MgCl_2$, 0.01 M KCl, and 0.1 mM $FeSO_4$-$(NH_4)_2SO_4$. The liquid medium employed contained the same constituents with the agar omitted.

Preparation of cell suspensions. Cells were grown in 250 ml of liquid medium in a 2-liter flask for 16 hr at 25 C on a rotary shaker and were harvested by centrifugation at 16,000 × *g* at 4 C. Unless otherwise stated, the cells were washed three times by resuspension in and centrifugation from volumes of 0.05 M $MgSO_4$ equal to the volume of the growth medium.

Preparation of cell envelopes. The cell envelopes were prepared by a slight modification of the procedure described by Buckmire and MacLeod (4). Cells were grown and harvested as described above, but they were washed in 0.5 M NaCl. A suspension of the cells adjusted to contain the equivalent of 33 to 35 mg (dry weight) of cells per ml in a total volume of 20 ml was placed in a polypropylene tube and frozen by using liquid air. The suspension was thawed at room temperature, diluted with 0.5 M NaCl to a volume of 200 ml, and centrifuged at 3,500 × *g*. The supernatant fluid was removed and the sedimented matter was resuspended in 20 ml of 0.5 M NaCl. The freezing and thawing treatment was repeated once more by which time all the cells had lost their contrast, as indicated by phase-contrast microscopy. The envelopes were washed by repeated resuspension in and centrifugation from 0.5 M NaCl until the optical density (OD) of the washings measured at 260 nm had dropped essentially to zero. Examination of thin sections by electron microscopy revealed large fragments of envelopes, some in the shape of whole cells containing variable but small amounts of nuclear material and no evidence of ribosomes.

OD changes. For these measurements, a suspension of appropriately washed cells was added in 0.1-ml volumes to 9.9 ml of the test solution, and the OD of the resulting suspension was measured at intervals with a Coleman Junior spectrophotometer at 525 nm. The final cell concentration in each suspension contained the equivalent of 0.33 to 0.35 mg (dry weight) of cells per ml. For the measurement of OD changes in suspensions of envelopes, 0.05-ml samples of envelope suspension were added to 1.5-ml volumes of the test solution contained in 0.5-cm cuvettes. Absorbancy changes were measured with a Zeiss PMQ II spectrophotometer. The cell envelope suspension contained envelopes equivalent to 3 mg (dry weight) of cells per ml.

Intracellular fluid volume. Intracellular fluid volume was measured by a modified thick-suspension technique essentially as described previously (19), except that ^{14}C-inulin was used in place of ^{14}C-carboxypolyglucose for the measurement of the extracellular fluid volume, and the cells were suspended in a solution containing 0.1 M tris(hydroxymethyl)aminomethane (Tris) buffer (*p*H 7.2), 0.05 M $MgSO_4$, and various concentrations of NaCl.

Intracellular Na^+ and Cl^-. Intracellular Na^+ and Cl^- concentrations were determined by procedures described previously (19), except that the cells were suspended in a solution containing 0.01 M Tris buffer (*p*H 7.2), 0.05 M $MgSO_4$, and concentrations of NaCl as indicated in the text. Corrections were made for ions present in the extracellular fluid using ^{14}C-inulin for the measure of the extracellular fluid volume.

Inulin and ^{22}Na space. Inulin space in a packed cell preparation was taken as the extracellular fluid volume, determined as described previously (19) but using ^{14}C-inulin in place of ^{14}C-carboxypolyglucose. The cells were suspended in the same medium used to determine the intracellular ion concentrations. ^{22}Na space was determined on another sample of the same cell suspension used to measure the inulin space. ^{22}Na (specific activity about 22 mc/mM) was introduced as the chloride salt into the cell suspension at a concentration of 0.015 μc/ml. The cells were separated by centrifugation and the supernatant fluid was removed. The radioactivity of the supernatant fluid and of the separated cells was determined by using a well-type scintillation counter. From the radioactivity of the separated cells, it was possible to calculate the volume of supernatant fluid to which this ^{22}Na activity corresponded. This volume was considered to be the ^{22}Na space.

Na^+ determination. For the determination of Na^+, all samples were digested by a modification of the procedure of Sanui and Pace (18). Samples were digested by heating first with nitric and then with perchloric acid in Vycor-brand Kjeldahl flasks until the samples became colorless, after which the residual perchloric acid was removed by evaporation under partial vacuum. Na^+ analyses were performed by using a flame photometer attachment for a Zeiss PMQ II spectrophotometer.

Cl^- determination. Samples were prepared and analyzed for Cl^- by essentially the procedures of Wilson and Ball (21). Packed-cell preparations were mixed with an excess of a standard solution of $AgNO_3$; concentrated HNO_3 was added and the mixture was heated for 45 min over a bath of boiling water. In the case of supernatant solutions, a standard solution of $AgNO_3$ was added directly to the samples to be analyzed, followed by concentrated HNO_3. The Cl^- content of the cells and of the supernatant solutions was determined by back titrating the excess $AgNO_3$ with a standard solution of NH_4SCN, using $FeSO_4$ $(NH_4)_2SO_4$ as indicator.

Radioactivity measurements. For the measurement of ^{14}C activity, 0.1-ml volumes of the various solutions were pipetted onto circles of Whatman no. 1 filter paper which were then dried. Each filter-paper disc was placed in a vial and covered with a liquid scintillation mixture (5); radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.). ^{22}Na measurements were made with a Picker well-type scintillation counter. Care was taken to maintain constant the geometry of all samples. In all cases, a

total of at least 10,000 counts was accumulated on each sample to reduce the probable counting error to less than 1%.

RESULTS

Optical effects produced by various solutes. When equal samples of a suspension of marine pseudomonad B-16 that had been washed and suspended in 0.05 M $MgSO_4$ were added to equal volumes of solutions differing in solute composition, the resulting suspensions were found to vary in OD, depending on the kind and concentration of the solutes present. In these experiments, the final suspensions were made in a basal, buffered salt solution to which various additional solutes were added. Two distinct phases of OD change could be distinguished (Table 1). The first phase was an initial rapid increase in OD, complete within seconds after suspension of the cells. The second phase was a much slower decrease in OD which occurred after the peak OD had been reached. It is evident that the salts of various monovalent cations and sucrose were effective in producing a response. In the case of NaCl, the maximal increase in absorbancy was obtained at a concentration of 200 mM. $MgSO_4$ was also effective in producing a response (Table 2). It is of interest that combinations of $MgSO_4$ and NaCl produced a greater initial increase in turbidity than did $MgSO_4$ alone.

The rapid increase in turbidity of suspensions of the marine pseudomonad in response to the addition of solutes followed by a slow decrease is typical of the response obtained in suspensions of a number of gram-negative bacteria (1, 2, 14, 15, 17).

Requirement for K^+ for the second-phase optical change. The basal salt mixture used as a suspending medium in these initial studies contained K^+ and Mg^{++} at levels optimal for the growth of this organism (12, 13). Omission of the basal level of KCl from the suspending solution had no effect on the first-phase optical change or on the increase in absorbancy of the suspension on addition of solutes, but it almost completely eliminated the second-phase optical change, the subsequent slow decrease in absorbancy after the initial increase (Table 3). Other experiments have shown that omission of the basal level of Mg^{++} from the suspending solution had no effect on either phase of the optical response to added solutes.

Correlation of absorbancy changes with changes in intracellular fluid volume. The optical changes which occur in bacterial suspensions on addition of solutes are assumed to be manifestations either of changes in size of the organism or of different degrees of plasmolysis of the cells (1, 2, 6). Bernheim (2) concluded that OD changes in suspensions of *Pseudomonas aeruginosa* corresponded to changes in size of the cells as observed by electron microscopy of shadowed preparations. Lehninger (10) obtained a good correlation between OD change and volume change measured gravimetrically in rat liver mitochondria.

In the present study, changes in OD of suspensions of the marine bacterium were related to cell volume measured directly. The results in Fig. 1 show that as the salt concentration increased to 200 mM the intracellular fluid volume

TABLE 1. *Effect of various solutes on the OD changes occurring in suspensions of cells of marine pseudomonad B-16*

Solute[a]	Concn (mM)	OD at minutes in suspension[b]		
		0.5	10	60
None		0.325	0.280	0.270
NaCl	50	0.360	0.310	0.305
NaCl	100	0.385	0.295	0.300
NaCl	200	0.420	0.350	0.330
NaCl	500	0.400	0.400	0.370
KCl	200	0.435	0.360	0.320
LiCl	200	0.440	0.390	0.370
NH_4Cl	200	0.430	0.380	0.365
Sucrose	400	0.380	0.350	0.335
Raffinose	400	0.330	0.305	0.290

[a] Added to a salt solution containing 0.01 M Tris buffer (*p*H 7.2), 0.05 M $MgSO_4$, and 0.01 M KCl.

[b] Measured at 525 nm. The experiment was started by adding equal samples of a washed suspension of cells to cuvettes containing the test medium.

TABLE 2. *Effect of different concentrations of $MgSO_4$ on the OD changes in suspensions of cells of the marine pseudomonad*

Concn of solutes (mM)[a]		OD at minutes in suspension			
$MgSO_4$	NaCl	0.5	10	30	60
1	0	0.250	0.235	0.220	0.220
50	0	0.350	0.320	0.300	0.295
100	0	0.375	0.355	0.340	0.335
200	0	0.405	0.395	0.365	0.365
1	200	0.430	0.385	0.360	0.360
50	200	0.450	0.395	0.360	0.365
100	200	0.445	0.385	0.360	0.360
200	200	0.460	0.405	0.400	0.395

[a] Suspending medium also contained 0.01 M Tris buffer (*p*H 7.2) and 0.01 M KCl.

TABLE 3. *Effect of K^+ on OD changes in suspensions of cells of marine pseudomonad B-16*

Solute[a]	Concn (mM)	KCl	OD at minutes in suspension		
			0.5	10	60
None.....		+	0.330	0.280	0.270
		−	0.325	0.325	0.325
NaCl.....	200	+	0.435	0.340	0.325
	200	−	0.440	0.430	0.420
KCl.....	200	+	0.430	0.360	0.320
	200	−	0.430	0.355	0.315
LiCl.....	200	+	0.440	0.390	0.370
	200	−	0.440	0.430	0.415
Sucrose....	400	+	0.380	0.350	0.335
	400	−	0.380	0.375	0.355

[a] Added to a suspending medium containing 0.01 M Tris buffer (*p*H 7.2) and 0.05 M $MgSO_4$. KCl added where indicated at 0.01 M.

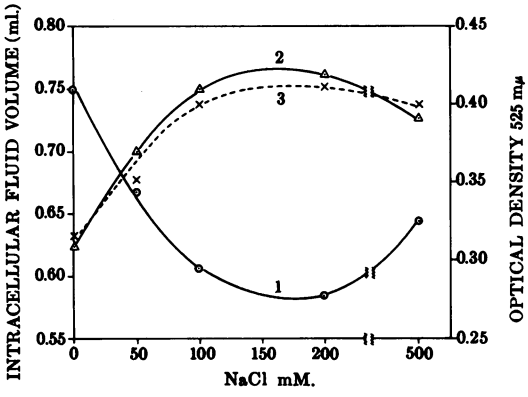

FIG. 1. *Correlation between OD changes in suspensions of marine pseudomonad B-16 and changes in intracellular fluid volume in response to NaCl in the suspending medium. Curve 1 represents intracellular fluid volume; curve 2, OD after 0.5 min; and curve 3, OD after 60 min.*

decreased. Above the 200-mM NaCl concentration, the intracellular fluid volume increased slightly. The turbidity of the suspension showed an opposite effect, increasing with increasing salt concentration to 200 mM NaCl and then decreasing. The turbidity values obtained initially remained essentially unchanged after 60 min of incubation. This result was obtained since K^+ was omitted from the suspending medium in this experiment. Thus, the increase in OD of the suspension of marine pseudomonad B-16 corresponded to a decrease in intracellular fluid volume of the cells, and hence to a decrease in the size of the cells. Since it has been calculated that the swelling or contraction caused by the uptake or release of water from particles of the dimensions and composition of bacteria causes a change in light absorbancy which is inversely proportional to the two-thirds power of the volume of the particle (8), one may reasonably conclude that the increase in OD of suspensions of the marine pseudomonad in the presence of NaCl is due to a decrease in the volume of the cells.

Intracellular Na^+ and Cl^- concentration. It is generally assumed that the initial increase in turbidity of a suspension of cells upon addition of NaCl is due to an osmotic phenomenon. NaCl is assumed to be a nonpenetrating or slowly penetrating solute which causes the cells to shrink by increasing the osmotic pressure of the medium. Previous studies have shown that Na^+ assumes the same concentration inside the cells of this organism as prevails in the suspending medium (19). To establish whether this still applied under the conditions used in the present studies and to determine the disposition of the anion involved, the intracellular Na^+ and Cl^- concentrations in the marine pseudomonad were determined when the cells were suspended in the presence and in the absence of added NaCl (Table 4). In the absence of added NaCl, there was a small amount of Na^+ and Cl^- present as a contaminant in the suspending medium. Under these circumstances, there was more Na^+ in the cells than in the medium. This small amount represents Na^+ tightly bound to the cells (R. C. Gordon and R. A. MacLeod, *unpublished data*). Under the same conditions, Cl^- was also somewhat higher in the cells than in the medium. When NaCl was added to the suspension at a concentration of 200 mM, the concentration of Na^+ in the cells became very nearly the same as in the medium. The Cl^- concentration in the cells was somewhat less than in the medium.

Further support for the conclusion that an NaCl gradient is not maintained across the membrane of this organism was obtained by measuring the volume penetrated by ^{22}Na (^{22}Na space) in cell preparations of the marine pseudomonad. In this experiment, the volume occupied by ^{22}Na in a packed-cell preparation was compared with the total fluid volume and with the extracellular fluid volume as determined by using ^{14}C-inulin. The results (Table 5) show that when no unlabeled NaCl was added to the suspending medium the ^{22}Na space exceeded the total fluid volume, suggesting that some ^{22}Na was exchanged for Na^+ bound to the cells. When

TABLE 4. *Intracellular Na^+ and Cl^- concentrations in cells of marine pseudomonad B-16 in the presence and in the absence of NaCl*[a]

Suspending medium[b]			Intracellular concn	
NaCl added	Na^+ found	Cl^- found	Na^+	Cl^-
0	0.1 ± 0.0	14.6 ± 0.0	1.0 ± 0.1	29.0 ± 0.9
200	204 ± 1.0	222 ± 2.0	219 ± 4.0	175 ± 8.0

[a] Values are expressed as millimolar units. Analyses represent the average and average deviations of triplicate determinations.

[b] Contained, in addition, 0.01 M Tris buffer (*p*H 7.2) and 0.05 M $MgSO_4$.

NaCl was added at a level of 200 mM, the amount of bound Na^+ became negligible compared to the total Na^+ present. The ^{22}Na space determined under these conditions was quite similar to the total available fluid space in the cell preparations.

Effect of metabolic inhibitors. An energy-dependent water extrusion mechanism has been detected in mitochondria (11), chloroplasts, and photosynthetic bacteria (16). If such a mechanism were present in the marine pseudomonad and required salts for activation, the initial decrease in intracellular fluid volume which occurs upon the addition of salts could be explained. As a test for the operation of such a system, the effect of metabolic inhibitors on the production of optical changes by salts was examined. The results (Table 6) show that none of the inhibitors tested prevented the initial increase in turbidity which occurred upon the addition of 200 mM NaCl to the suspension. The subsequent slow decrease which could occur because K^+ was present, however, was prevented or retarded by the presence of the inhibitors. In a further effort to influence the extent or rate of the initial increase in turbidity upon the addition of salts, the cells were preincubated with the inhibitors in the basal suspending medium for 1 hr before adding the 200 mM NaCl. No change in response was obtained.

Examination of cytoplasmic components. Whitfield and Murray (20) reported that chromatin aggregation could occur in cells of *Shigella dysenteria*, *Bacillus cereus*, and *Escherichia coli* upon immersion of agar blocks in which the organisms were growing into a solution of high (1.7 M) NaCl concentration. The cells were stained with a nuclear stain and examined with a light microscope. At the high salt concentration, the nuclear material of the cells aggregated into a body which looked like an axial filament. A similar phenomenon was observed by Johnson and Gray (7) to occur in the marine organism *Achromobacter fischeri*.

TABLE 5. *Comparison of the total fluid volume and the ^{22}Na space in packed cell preparations of marine pseudomonad B-16 in the presence and in the absence of unlabeled NaCl*

NaCl in suspending medium[a]	Total fluid vol	Inulin space	^{22}Na space
mM	*ml*	*ml*	*ml*
0	1.39	0.66	4.08
200	1.17	0.57	1.034

[a] Contained, in addition, 0.01 M Tris buffer (*p*H 7.2) and 0.05 M $MgSO_4$.

TABLE 6. *Effect of metabolic inhibitors on OD changes in suspensions of marine pseudomonad B-16*

Inhibitors[a]	Concn (M)	OD at minutes in suspension		
		0.5	10	60
Without added NaCl				
None		0.330	0.270	0.260
DNP	2×10^{-4}	0.345	0.360	0.325
KCN	2×10^{-2}	0.360	0.300	0.275
NaN_3	2×10^{-2}	0.380	0.350	0.340
PCMB	2×10^{-3}	0.340	0.320	0.310
$HgCl_2$	2×10^{-5}	0.320	0.325	0.325
With 0.2 M NaCl				
None		0.430	0.365	0.340
DNP	2×10^{-4}	0.440	0.405	0.400
KCN	2×10^{-2}	0.440	0.415	0.400
NaN_3	2×10^{-2}	0.440	0.410	0.410
PCMB	2×10^{-3}	0.440	0.420	0.350
$HgCl_2$	2×10^{-5}	0.430	0.430	0.370

[a] Added to a suspending medium containing 0.01 M Tris buffer (pH 7.2), 0.05 M $MgSO_4$, and 0.01 M KCl. DNP = 2,4-dinitrophenol; PCMB = *p*-chloromercuribenzoate.

The possibility was considered that the OD increase which occurred upon the addition of 200 mM NaCl might be due to chromatin aggregation. Staining of the nuclear material was carried out on cells of the marine pseudomonad according to the procedure of Whitfield and Murray (20). No evidence of chromatin aggregation was obtained either in the presence or in the absence of NaCl at the concentration tested.

Effect of composition of washing solution on optical effects. The OD changes observed were obtained with cells washed with 0.05 M $MgSO_4$ solution. Suspensions of cells washed in this way showed optical responses similar to those reported in the literature for terrestrial species. It was observed, however, that cells responded differently if washed with 0.5 M NaCl or with a solution of NaCl, KCl, and $MgSO_4$ at concentrations of the salts permitting optimal growth of the organism. A comparison of the effect of different washing solutions on subsequent optical density changes in suspensions of the marine bacterium is presented in Table 7. Equal numbers of cells washed in the three washing solutions were placed in the basal suspending medium containing various concentrations of NaCl. Cells washed in 0.5 M NaCl or in the salt mixture showed a relatively small initial response to added NaCl, as compared to cells washed in $MgSO_4$ solution, and a higher optical density at 500 mM NaCl than at 200. Second-stage optical effects tended to show increases rather than decreases in optical density with time. It is of considerable interest that after 60 min of incubation all suspensions appeared to be approaching the same optical density at each level of NaCl tested, irrespective of how the cells were washed.

TABLE 7. *Effect of different washing solutions on OD changes in suspensions of marine pseudomonad B-16 in response to NaCl*

Washing solutions	NaCl[a] (mM)	OD at minutes in suspension		
		0.5	30	60
0.05 M $MgSO_4$	0	0.340	0.325	0.315
	50	0.390	0.330	0.330
	100	0.450	0.370	0.360
	200	0.520	0.380	0.370
	500	0.450	0.435	0.420
0.5 M NaCl	0	0.280	0.290	0.295
	50	0.275	0.285	0.285
	100	0.275	0.305	0.310
	200	0.300	0.320	0.325
	500	0.370	0.375	0.375
0.2 M NaCl	0	0.255	0.300	0.300
0.01 M KCl	50	0.260	0.305	0.305
0.05 M $MgSO_4$	100	0.275	0.325	0.325
	200	0.305	0.340	0.335
	500	0.380	0.390	0.390

[a] Added to a suspending medium containing 0.01 M Tris buffer (pH 7.2), 0.05 M $MgSO_4$, and 0.01 M KCl.

Effects of salts on isolated envelopes. Suspensions of isolated envelopes of the marine pseudomonad were tested to determine their capacity to respond optically to different concentrations of salts (Fig. 2). With Mg^{++} present in the suspending solution, somewhat higher optical densities were obtained in the absence than in the presence of NaCl. When Mg^{++} was omitted, however, an increase in turbidity occurred with increasing NaCl concentration, in a manner quite comparable to the responses obtained with whole cells.

DISCUSSION

The second phase of the optical changes occurring when the solute concentration of the suspending medium is increased could be separated from the first, in the case of the marine pseudomonad, either by removing K^+ from or adding metabolic inhibitors to the suspending medium. Thus, the first-phase change, the initial increase in turbidity produced by added solutes, could be maintained and the factors giving rise to it could be examined.

When NaCl was used to increase the OD of the suspension, it was found that up to a concentration of 200 mM the space not occupied by inulin in the suspension was reduced in proportion to

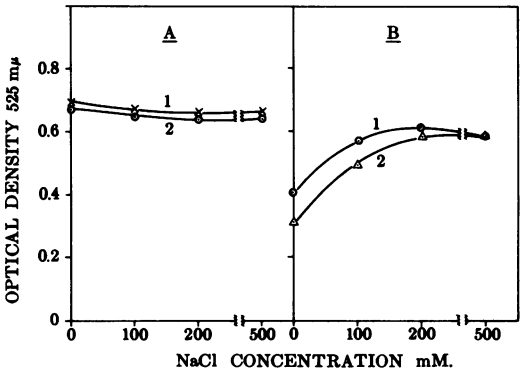

FIG. 2. *OD changes in suspensions of isolated envelopes of marine pseudomonad B-16 in response to NaCl in the presence and in the absence of $MgSO_4$. (A) 0.05 M $MgSO_4$ present. (B) $MgSO_4$ absent. Curve 1 represents OD after 0.5 min; curve 2, OD after 10 min.*

the increase in NaCl concentration. Studies of the comparative penetrability of inulin and sucrose into cells of this marine pseudomonad (F. L. A. Buckmire and R. A. MacLeod, *unpublished data*) suggest that the permeability barrier to inulin in these cells is the outer layer of the cell wall, whereas that to sucrose is the cytoplasmic membrane. Thus, decreases in the space not occupied by inulin in the suspension indicate a decrease in the volume of the whole cell rather than an increase in the degree of plasmolysis. Cells washed with 0.05 M $MgSO_4$, however, do show evidence of plasmolysis both in the presence and in the absence of NaCl when examined by phase-contrast microscopy. This latter phenomenon is presently under investigation.

Direct measurement of intracellular Na^+ and Cl^- showed that the two ions had assumed essentially the same concentration inside the cells as prevailed in the medium. This confirms earlier findings (19) and is supported by measurement of the ^{22}Na space in the suspension. Thus, the extent of increase in OD which occurred reflected the degree to which the cells had shrunk; yet no NaCl gradient was maintained across either the cell wall or the cytoplasmic membrane. The possibility that the increase in OD could have been caused by an initial difference in NaCl concentration between the inside and the outside of the cell due to a slow penetration of the salt was ruled out, since, if this had been the case, one would have expected a gradual decrease in turbidity again as the salt concentration reached equilibrium across the permeability barrier. In the absence of K^+, no such decrease occurred. The initial increase in turbidity was maintained over the period necessary to establish that the internal Na^+ and Cl^- had already reached the concentration prevailing in the medium. Thus, NaCl could not have caused the cells to shrink by osmotic action.

Since metabolic inhibitors had no effect on the shrinkage of cells caused by NaCl, it seems unlikely that the salt had activated a type of energy-dependent contractile mechanism such as has been described in mitochondria (11) and in photosynthetic bacteria (16). Furthermore, no evidence of aggregation of nuclear material in the presence of NaCl could be obtained. Thus, by a process of elimination it seemed most likely that NaCl produced its effects through interaction with the cell envelope of the organism. Support for this conclusion was obtained when it was found that NaCl caused increases in OD of suspensions of isolated cell envelopes of the organism. Conditions were not exactly the same as those used with whole cells, however, since in the presence of 0.05 M $MgSO_4$ no increase in turbidity of the envelope suspensions occurred upon the addition of NaCl.

When cell envelopes of this organism are suspended in distilled water, soluble, nondialyzable material appears in solution (4). The release of this material from the envelopes can be prevented by the addition of salts to the suspending medium. The material released at low salt concentration consists of complex macromolecules or aggregates of macromolecules composed of carbohydrate, lipid, and protein. (F. L. A. Buckmire and R. A. MacLeod, *unpublished data*). Evidence has been obtained that these macromolecules are highly electronegative. Low concentrations of Mg^{++} prevent the release of these macromolecules from the cell envelope without screening all the negative charges. Thus it is likely that when cells of this organism are washed and suspended in 0.05 M $MgSO_4$ there is sufficient Mg^{++} to form cross-links between the macromolecules, but insufficient to screen the negative charges completely. The macromolecules might thus be expected to repel one another sufficiently to stretch the envelope and cause the cells to swell. Higher concentrations of salts could screen the rest of the negative charges, thereby causing the envelope to contract and the cells to shrink. It is considered significant that NaCl at a concentration of 200 mM produced maximal shrinkage of the cells since this is the NaCl concentration optimum for preventing leakage of intracellular solutes from the cells (22) and for obtaining optimal growth of the organism (5). Above a concentration of 200 mM, NaCl caused the cells to swell again and to leak intracellular solutes (22), and it inhibited growth (5). The reasons for this are obscure at present.

The failure of isolated envelopes to duplicate the response of whole cells to NaCl in the presence of Mg^{++} may well be due to the fact that in whole cells there is an outward thrust produced by internal osmotic pressure which could balance a tendency of Mg^{++} cross-links to cause envelope contraction by mechanical means.

The differences in response of suspensions of cells to added NaCl, depending on the salt solutions used to wash the cells, may possibly be related to the fact that cells of this organism leak small molecules when suspended in 0.05 M $MgSO_4$ but not when the suspending medium contains an appropriate concentration of Na^+ (22). Potassium, which is ordinarily present in these cells at a concentration of 0.2 M, is lost from the cells when they are washed with 0.05 M $MgSO_4$ (V. S. Srivastava, P. T. S. Wong, and R. A. MacLeod, *unpublished data*). As suggested by Kuczynski et al. (9) in connection with

gram-positive bacteria which have lost their amino acid pool, perhaps cells which have lost small molecules have a lower internal osmotic pressure and, hence, are able to contract to a greater extent than cells with their intracellular solutes intact.

Other salts, like NaCl, would be expected to cause cell shrinkage by envelope contraction in the same manner as NaCl. Sucrose and raffinose, however, should behave differently. Since sucrose does not penetrate the cytoplasmic membrane of this organism (F. L. A. Buckmire and R. A. MacLeod, *unpublished data*), its capacity to shrink the cells can be satisfactorily explained by its osmotic action.

The second-phase optical changes occurring in suspensions of cells washed with 0.05 M $MgSO_4$ required K^+ and were prevented by metabolic inhibitors. Evidence to be presented elsewhere will indicate that, as in the case of *E. coli* (3, 17), these changes result from the energy-dependent transport of K^+ and other metabolites, when present, into the cells.

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