JOURNAL OF BACTERIOLOGY, Mar. 1970, p. 1014-1026
Vol. 101, No. 3

Stability and Comparative Transport Capacity of Cells, Mureinoplasts, and True Protoplasts of a Gram-Negative Bacterium[1, 2]

I. W. DE VOE,[3] J. THOMPSON, J. W. COSTERTON, AND ROBERT A. MACLEOD

Department of Microbiology, Macdonald College of McGill University, and Marine Sciences Center, McGill University, Montreal, Quebec, Canada

Received for publication 18 August 1969

The outer layers of the cell envelope of a pseudomonad of marine origin were removed by washing the cells in 0.5 M NaCl followed by suspension in 0.5 M sucrose. The term mureinoplast has been suggested for the rod-shaped forms which resulted from this treatment. As previously established, these forms lacked the outer cell wall layers but still retained a rigid peptidoglycan structure. Mureinoplasts remained stable if suspended in a balanced salt solution containing 0.3 M NaCl, 0.05 M $MgSO_4$, and 0.01 M KCl but, unlike whole cells, lost ultraviolet (UV)-absorbing material if suspended in 0.5 M NaCl or 0.05 M $MgCl_2$. Sucrose added to the balanced salt solution also enhanced the loss of UV-absorbing material. Addition of lysozyme to suspensions of mureinoplasts in the balanced salt solution produced spherical forms which, by electron microscopy and the analysis of residual cell wall material, appeared to be true protoplasts. Only undamaged mureinoplasts, as judged by their capacity to fully retain α-aminoisobutyric acid, were capable of being converted to protoplasts. Protoplasts and undamaged mureinoplasts retained 100% transport capacity when compared to an equal number of whole cells. The Na^+ requirement for transport of α-aminoisobutyric acid and the sparing action of Li^+ on this Na^+ requirement were the same for both protoplasts and whole cells. These observations indicate that, in this gram-negative bacterium, the cell wall does not participate in the transport process though it does stabilize the cytoplasmic membrane against changes in porosity produced by unbalanced salt solutions. The results also indicate that the requirements for Na^+ for transport and for the retention of intracellular solutes are manifested at the level of the cytoplasmic membrane.

The lack of a suitable technique for the removal of the cell wall from gram-negative bacteria has precluded investigation into the specific functions of the cytoplasmic membrane of these organisms. In a recent review, Rothfield and Finkelstein (28) discussed the advantages to be gained by the complete removal of the cell wall from intact gram-negative cells; other investigators have also emphasized such advantages (17, 25, 30). Recent findings in this laboratory have provided a method for the removal of the cell wall components of a marine pseudomonad and thus for the formation of true protoplasts of a gram-negative bacterium (10).

[1] Issued as Macdonald College Journal Series No. 600.

[2] Presented in part at the Nineteenth Annual Meeting of the Canadian Society of Microbiologists, Ottawa, Ontario, 5-7 June 1969.

[3] Present address: Biology Department, Aurora College, Aurora, Ill.

Gram-negative marine bacteria generally require Na^+ for growth and metabolism (21) and have a requirement for cations for the maintenance of cell integrity (8, 11, 22). With the exception of these requirements, gram-negative marine bacteria closely resemble many of their terrestrial counterparts structurally (34), biochemically (12; C. Forsberg, J. W. Costerton, and R. A. MacLeod, *unpublished data*), and physiologically (21).

The classical studies of Gale (16) and others have shown that bacteria have the capacity to accumulate amino acids (5, 6, 26) and sugars (3, 15, 20, 27) against considerable concentration gradients. This accumulation process is dependent upon an energy source (1, 24, 31, 36) which is coupled in some as yet undefined way to substrate-specific carriers or permeases (9, 19, 20). Investigations with such gram-positive organisms

as *Streptococcus faecalis* (4, 23), *S. faecium* (6), and *S. diacetilactis* (32) indicate that the transport systems are located in the cytoplasmic membrane. The cell wall did not seem to participate in the transport process, although Mora and Snell (23) found two major differences in the properties of an amino acid transport system of *S. faecalis* after cell wall removal.

Although studies on the transport of amino acids and galactosides have been carried out with gram-negative bacteria, specific localization of the transport systems has been impossible because of the lack of suitable methods for the complete removal of the cell wall of these organisms. Anraku (2) has pointed out that no direct evidence exists for the exact location of these transport systems. As a result of studies on proline uptake by *Escherichia coli*, Kaback and Stadtman did suggest that amino acid uptake is a functional characteristic of the cytoplasmic membrane in gram-negative species (18). The cell system upon which they based their conclusion, however, was not cell wall-free. Brown, on the other hand, decided that the outer membrane of the cell envelope of a marine pseudomonad participates in transport processes (7).

Studies in this laboratory have shown that a marine pseudomonad transports amino acids, sugars, and other compounds by a Na^+-dependent process (13, 37). Protoplasts of this organism can be prepared and were found to be capable of accumulating α-aminoisobutyric acid-*1*-^{14}C (^{14}C-AIB) if NaCl was present (10). Under the conditions employed, the protoplasts appeared to be less than one-half as active as whole cells of the organism in concentrating the amino acid analogue. A study was therefore undertaken to determine whether the lower capacity of these protoplasts to transport was due to the conditions employed in preparing and testing the protoplasts or whether the outer layers of the cell envelope were necessary for maximum transport activity.

MATERIALS AND METHODS

Organism. The organism used and designated marine pseudomonad B-16 was originally isolated from a marine clam and has been classified as a *Pseudomonas* species type IV. It has been deposited in the American Type Culture Collection (ATCC 19855) and the National Collection of Marine Bacteria (NCMB 19). Studies on the nutrition and metabolism of this organism have been reported in some detail in previous communications, the most recent being Wong et al. (37).

Medium and growth conditions. The media used for the maintenance of stock cultures and for growth have each been described previously (10). The procedure used to grow the cells was designed to produce cultures in the exponential phase of growth in a reasonably high yield. Cells from an agar slant culture of the organism were inoculated into 10 ml of broth medium contained in a 50-ml Erlenmeyer flask and incubated for 8 hr. The contents of this flask were then used to inoculate a 250-ml volume of broth medium contained in a 2-liter flask, which was in turn incubated for 5 hr. A 40-ml amount of this latter culture was used to inoculate another 250 ml of broth medium in a 2-liter flask which was also incubated for 5 hr. The cells from this last culture were harvested by centrifugation for subsequent studies. All broth cultures were incubated on a rotary shaker at 25 C.

Preparation of mureinoplasts. The term mureinoplast will be used to describe a gram-negative bacterium which has had the outer layers of its cell wall removed and is surrounded only by its cytoplasmic membrane and peptidoglycan [murein (35)]. Procedures for producing such a form from marine pseudomonad B-16 have been described (10). A slight modification of these procedures was used in the present study. Cells were harvested from the growth medium by centrifugation at 16,000 $\times$ *g* at 4 C and washed three times by resuspension in and centrifugation from volumes of 0.5 M NaCl equal to the volume of the growth medium. The washed cells were resuspended in this volume of 0.5 M sucrose, and the suspension was incubated at 25 C for 30 min on a rotary shaker. The cells were harvested by centrifugation, resuspended in the same volume of 0.5 M sucrose, and centrifuged immediately. The mureinoplasts thus formed were tested for their stability in the presence of various solutes by resuspension in the appropriate solution at a density equivalent to 2 mg (dry weight) of whole cells per ml. In, some instances, the suspensions were centrifuged at 16 000 $\times$ *g* for 10 min, and the supernatant fluids were examined for the presence of ultraviolet (UV)-absorbing material by scanning over the range 220 to 300 nm by using a recording Unicam spectrophotometer.

Preparation of protoplasts. Mureinoplasts were suspended to a density equivalent to 6 mg (dry weight) of cells per ml in a solution containing lysozyme (Sigma Chemical Co., St. Louis, Mo.; grade 1, 3 $\times$ crystallized), 150 μg/ml; NaCl, 0.3 M; $MgSO_4$, 0.05 M; KCl, 0.01 M; tris(hydroxymethyl)aminomethane (Tris) buffer (Trizma HCl, Sigma Chemical Co.; *p*H 7.5), 1 mM. Suspensions were incubated for periods as long as 2 hr. Protoplast formation was followed with the aid of a phase-contrast microscope. Centrifugation and resuspension of the protoplasts formed were avoided because of their fragility. To determine the effect of omitting or varying the concentration of the various salts in the incubation medium on the stability of the protoplasts formed, mureinoplasts were suspended in the appropriate medium, lysozyme was added, and protoplasts were formed directly in the suspending solutions.

Preloading cells with ^{14}C-AIB for retention studies. Cells harvested from the growth medium were washed three times by resuspension in and centrifugation from a solution, referred to as complete salts solution, which contained 0.3 M NaCl, 0.01 M KCl, and 0.05 M

$MgSO_4$. The cells were suspended at a concentration equivalent to 1.6 mg (dry weight) of cells per ml in a solution containing complete salts, 0.05 M Tris phosphate buffer (*p*H 7.2), and 7.5×10^{-4} M ^{14}C-AIB (specific activity, 0.11 μc/μmole), which was then incubated for 30 min at 25 C. To measure retention of ^{14}C-AIB by the cells after various treatments and washing procedures, 0.3-ml samples of the suspension were filtered on a membrane filter (pore size, 0.45 μm; Millipore Corp., Bedford, Mass.). The cells retained on the filter were washed with 5 ml of complete salts solution.

Measurement of capacity to transport. Whole cells, mureinoplasts, or protoplasts were suspended at a concentration equivalent to 600 μg/ml (dry weight) of original whole cells in the incubation medium used to preload the cells, except that the ^{14}C-AIB concentration was increased to 1.5×10^{-3} M with the specific activity maintained the same. At appropriate intervals, 0.5-ml volumes of the suspension were filtered on a 0.45-μm membrane filter. The cells retained on the filter were in each case washed with 5 ml of complete salt solution. To determine the effect of Na^+ on transport, the NaCl concentration in the incubation medium was varied either alone or by adding combinations of NaCl and LiCl so that the total concentration of the two salts was maintained constant at 0.5 M.

Radioactivity determination. The filters, plus adhering cells, were placed in 20-ml, screw-cap scintillation vials, dried thoroughly with the aid of an infrared lamp, and covered with 5 ml of scintillation fluid containing 5 g of 2,5-diphenyloxazole (PPO) and 0.3 g of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP) per liter of toluene. Radioactivity in the samples was determined by using a Tri-Carb liquid scintillation spectrometer (Packard Instrument Co., Inc., Downers Grove, Ill.)

Quantitation of cell numbers. Direct counts were determined by using cell suspensions diluted 50-fold in the appropriate suspending medium and by using a haemocytometer with a 0.01-mm chamber depth. Each suspension was counted in quadruplicate with 200 to 300 cells counted over 16 haemocytometer squares for a single determination. Corrections for cell aggregates, i.e., pairs and triplets, were made by employing an aggregation factor calculated by examination of approximately 200 cells by phase-contrast microscopy at 1,000 diam.

The number of viable cells, i.e., those cells capable of producing a visible colony on solid medium in 48 hr, was determined by diluting cells in complete salts solution at various stages in the above procedures and spreading 0.1 ml of the various dilutions on the surface of growth medium solidified with 1.5% agar. Plates were incubated for 48 hr at 25 C. Colonies were counted at 24 and 48 hr.

Phase-contrast and electron microscopy. Phase microscopy of unfixed wet mounts was carried out with a Carl Zeiss photomicroscope equipped with phase attachments. The procedure for electron microscopy of thin sections has been outlined previously (10), except that (i) cells were prefixed in 0.6% glutaraldehyde in the appropriate suspending medium and (ii) the Ryter and Kellenberger procedure (29) was omitted because previous work had shown that it failed to yield intact protoplasts. Sections were examined in an AEI EM-6B electron microscope by using 60-kv electron acceleration voltage.

RESULTS

Conditions for formation of stable mureinoplasts. The procedure for the removal of the outer layers of the cell wall of this organism requires that cells be washed in 0.5 M NaCl and subsequently suspended in 0.5 M sucrose (10). The forms produced are surrounded by a cytoplasmic membrane and a peptidoglycan layer and are herein referred to as mureinoplasts. Such forms can be converted to protoplasts by treatment with lysozyme.

When whole cells of this organism are suspended in the presence of sufficient $MgSO_4$ to prevent lysis but in the absence of an appropriate concentration of NaCl, the cells lose low molecular weight intracellular solutes quickly and their viability more slowly (37). It was therefore necessary to determine to what extent the procedures used for the formation of mureinoplasts and subsequently protoplasts caused damage to the cytoplasmic membrane of the organism and whether steps could be taken to reduce or eliminate this damage.

Cells of the marine pseudomonad were harvested from the growth medium and washed three times with 0.5 M NaCl. The washings were examined for their content of UV-absorbing material by scanning over the spectrum 220 to 300 nm. No UV-absorbing material could be detected. The cells were then resuspended in 0.5 M sucrose and incubated for 30 min. The cells were centrifuged from the suspension, and the supernatant fluid was examined for UV-absorbing material. The results (Fig. 1; curve 1) show that a considerable amount of material absorbing at 220 nm had appeared in solution, but there was no evidence of material being released from the cells which absorbed at 260 nm. The cells were resuspended in 0.5 M sucrose a second time and centrifuged immediately. The second supernatant fluid (curve 2) showed much less absorption at 220 nm and, again, essentially no absorption at 260 nm. After a third resuspension in sucrose, material with an absorption peak at 260 nm began to appear in the supernatant fluid, and more of this was apparent after a fourth suspension. As a result of this and similar experiments, it was concluded that the cells should not be washed with 0.5 M sucrose more than once after the 30-min incubation period in the presence of sucrose. Such treatment meant that the cells had been in contact with the sucrose solution and in

the absence of salts for a total, including centrifugation time, of 1.5 hr. Comparison of cells in thin section by electron microscopy, before (Fig. 2A) and after the application of this procedure, shows that the cells were devoid of the outer double-track layer (Fig. 2B).

Since the next stage in the procedure involved the conversion of the mureinoplasts to protoplasts, it was considered necessary to maintain the mureinoplasts in a solution which contained sucrose as an osmotic stabilizer. Since the above results show that extended exposure to 0.5 M sucrose in the absence of salts caused the mureinoplasts to lose 260 nm-absorbing material, it seemed desirable to add back the appropriate salts to the suspending solution as soon as possible after the mureinoplasts had been formed. It was therefore surprising to find that mureinoplasts suspended in 0.5 M sucrose lost more, rather than less, 260 nm-absorbing material when salts were added to the suspension (Fig. 3). It is evident that resuspension of the mureinoplasts in 0.5 M sucrose made 0.5 M with respect to NaCl lost a small amount more 260 nm-absorbing material than cells resuspended in 0.5 M sucrose alone. The addition of complete salts to the sucrose solution caused an even greater loss, whereas the greatest loss occurred when the sucrose solution contained 0.05 M $MgCl_2$. Another interesting finding not shown was that resuspension of mureinoplasts in 0.5 M sucrose made 0.05 M with respect to Tris buffer (*p*H 7.8) resulted in a marked release of 260 nm-absorbing material.

If the mureinoplasts were centrifuged from the suspension in 0.5 M sucrose and resuspended in the complete salts solution alone (Fig. 4), no UV-absorbing material was released into the suspending medium. It can be seen, however, that if the mureinoplasts were suspended in either 0.05 M $MgCl_2$ alone or in 0.5 M NaCl alone very large amounts of UV-absorbing material appeared in the suspending solution, even more than had been released when sucrose was present. When whole cells of this organism were suspended in such solutions, to the same cell density, no 260 nm-absorbing material could be detected.

A more sensitive indicator of membrane damage than loss of 260 nm-absorbing material is the extent of leakage of a small molecule such as ^{14}C-AIB from the cells. Cells of the organism were preloaded with ^{14}C-AIB, converted to mureinoplasts, and resuspended in various solutions. The ^{14}C-AIB retained by the mureinoplasts was determined after 40 min of incubation. The results (Table 1) show that mureinoplasts resuspended either in complete salts or in complete salts modified to contain 0.5 M NaCl retained essentially 100% of their ^{14}C-AIB. If 0.5 M sucrose

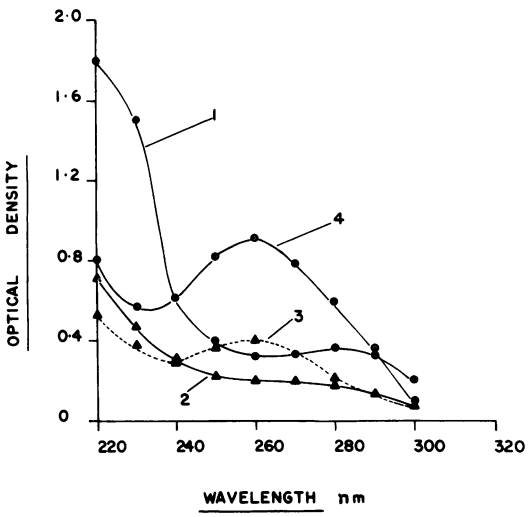

FIG. 1. *Ultraviolet absorption spectra of the supernatant fluid from cells suspended in 0.5 M sucrose. Curve 1, after 30 min of incubation; curve 2, after a second resuspension in 0.5 M sucrose; curve 3, after a third resuspension; curve 4, after a fourth resuspension.*

was included in the solution of modified complete salts, retention was reduced to 39%. Various other combinations of salts and sucrose were tested, but none was as effective as the complete salts in preventing the loss of ^{14}C-AIB. It is also evident that mureinoplasts suspended in 0.5 M sucrose alone lost most of their ^{14}C-AIB. In contrast, cells in the same suspending medium lost very little 260 nm-absorbing material (Fig. 3).

Conditions for formation of stable protoplasts. Since a solution containing sucrose in combination with complete salts led to the loss of both 260 nm-absorbing material and ^{14}C-AIB from mureinoplasts suspended in it, it was obviously necessary to avoid the use of sucrose as an osmotic stabilizer in the formation of protoplasts. An effort was therefore made to prepare protoplasts from mureinoplasts by suspending the latter in a solution of complete salts containing 1 mM Tris buffer (*p*H 7.5) and adding lysozyme. The rate and extent of conversion of rods to spheres were monitored by phase-contrast microscopy. The rod-shaped mureinoplasts (Fig. 5A) were converted to spherical forms in 10 min (Fig. 5B). Unexpectedly, these spherical forms remained stable for over 1 hr (Fig. 5C). To determine whether these spherical forms were in fact protoplasts, thin sections of the preparation were examined in the electron microscope (Fig. 6). It is evident that no outer double-track material lay outside of the cytoplasmic membrane which surrounded these spherical forms. The conversion

FIG. 2. *Electron micrographs of thin sections of (A) a whole cell and (B) a mureinoplast. [Note the absence of the double-track wall structure in the mureinoplast (B).] The bar on all electron micrographs represents 0.1 μm. × 91,500.*

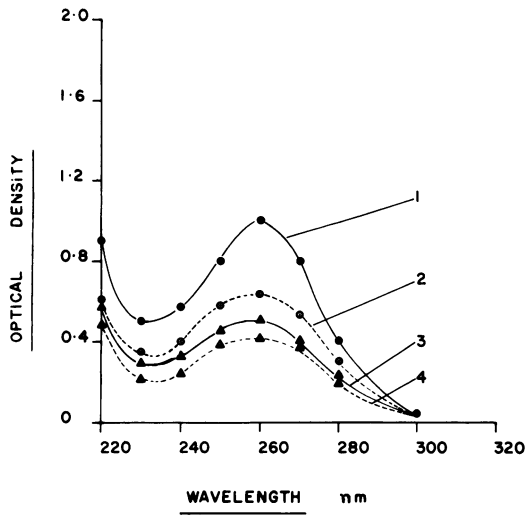

FIG. 3. *Ultraviolet absorption spectra of supernatant fluids from mureinoplasts suspended in: curve 1, 0.05 M $MgCl_2$ plus 0.5 M sucrose; curve 2, complete salts plus 0.5 M sucrose; curve 3, 0.5 M NaCl plus 0.5 M sucrose; curve 4, 0.5 M sucrose alone after third resuspension.*

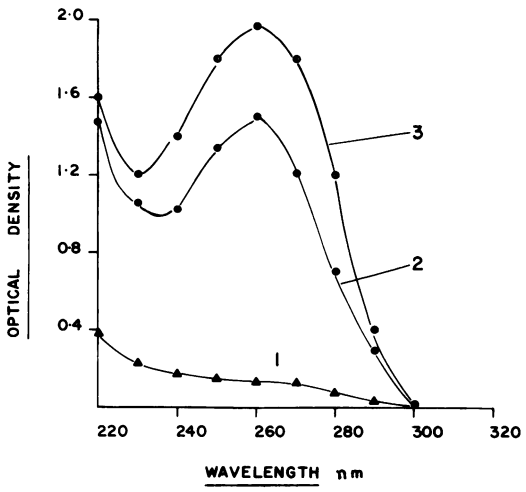

FIG. 4. *Ultraviolet absorption spectra of the supernatant fluid from mureinoplasts suspended in: curve 1, complete salts; curve 2, 0.5 M NaCl; curve 3, 0.05 M $MgCl_2$.*

of whole cells to mureinoplasts by the procedures described is essentially quantitative (Table 2). Mureinoplasts contain only a small fraction of the total hexosamine present in whole cells of this organism (C. Forsberg, J W. Costerton, and R. A. MacLeod, *unpublished data*) as a consequence of the loss of wall material occurring when mureinoplasts are formed. The residual hexosamine in the mureinoplasts was still further reduced by treatment of the mureinoplasts with lysozyme (Table 3). The lack of wall material associated with these spherical forms indicated that they were, in fact, protoplasts.

Quantitation of the conversion of cells to protoplasts. Whole cells of the organism were preloaded with ^{14}C-AIB and converted first to mureinoplasts and then to protoplasts. ^{14}C-AIB retention by the cells was followed during the manipulations by filtering equal portions of the suspensions at various stages and measuring the radioactivity retained by the cells on the filter. Simultaneous determinations of direct and viable counts were also made. The results (Fig. 7) show that the cells retained their viability during the washings with 0.5 M NaCl. Viability decreased to 0.1% within 30 sec after the cells were suspended in 0.5 M sucrose and further decreased to 0.001% after 30 min. This loss of viability was not due to cell lysis, since direct counts showed that the total numbers of cells remained unchanged and phase-dense during sucrose treatment. Separate experiments indicated that during this period of suspension in sucrose no 260 nm-absorbing material appeared in solution. When the mureinoplasts were suspended in the protoplasting medium, direct counts revealed that 25% of the mureinoplasts became protoplasts. The remaining 75% lysed during the conversion process. Measurement of ^{14}C-AIB retention during the manipulations indicated that during the washings with 0.5 M NaCl most of the labeled compound was retained by the cells. The greatest loss of radioactivity from the cells occurred during suspension in the sucrose solution. This loss can be explained in one of two ways. Either all cells lost a certain proportion of their ^{14}C-AIB during

TABLE 1. *Capacity of mureinoplasts to retain ^{14}C-AIB when suspended in various solutions for 40 min*

Constituents of suspending media	Per cent ^{14}C-AIB retained
Sucrose (0.5 M)	26
Sucrose (0.5 M), NaCl (0.2 M)	76
Sucrose (0.5 M), NaCl (0.5 M)	28
Sucrose (0.5 M), $MgCl_2$ (0.05 M)	26
Sucrose (0.5 M), NaCl (0.5 M), $MgCl_2$ (0.05 M)	49
Sucrose (0.5 M), NaCl (0.2 M), $MgCl_2$ (0.05 M)	39
Sucrose (0.5 M), NaCl (0.5 M), $MgCl_2$ (0.05 M), KCl (0.01 M)	39
NaCl (0.5 M), $MgCl_2$ (0.05 M), KCl (0.01 M)	96
NaCl (0.3 M), $MgCl_2$ (0.05 M), KCl (0.01 M)	100

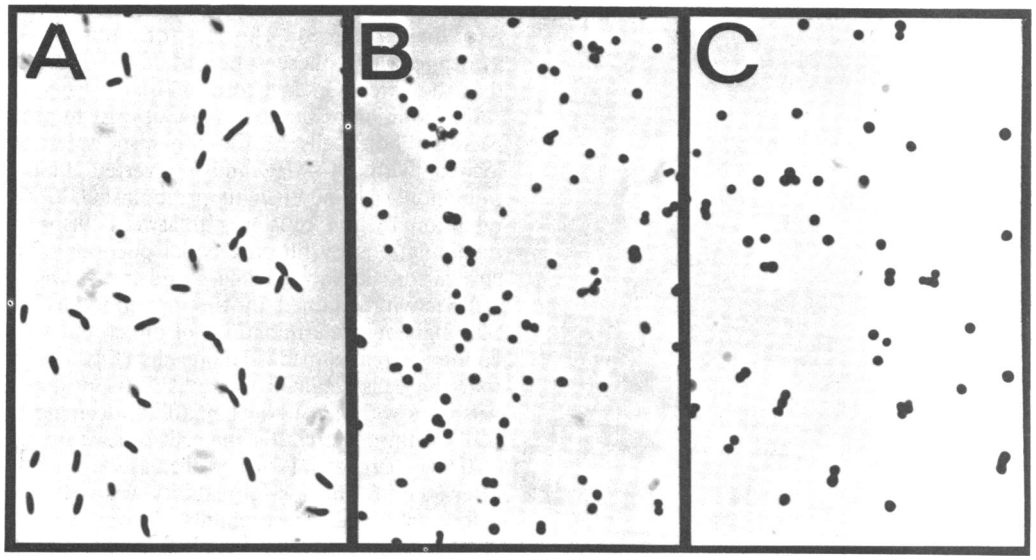

FIG. 5. *Phase-contrast micrographs of (A) mureinoplasts; (B) protoplasts 10 min after addition of lysozyme; and (C) protoplasts 80 min after the addition of lysozyme.* × *1,800.*

suspension in sucrose, or certain cells of the population lost all their ^{14}C-AIB. Strong evidence in favor of the latter possibility is the close correlation shown between the percentage of ^{14}C-AIB retained at the protoplast stage and the percentage of cells which survived the conversion to protoplasts. Thus, the protoplasts formed contained the same amount of ^{14}C-AIB per cell as the NaCl-washed whole cells from which they were derived. It may thus be concluded that only those mureinoplasts which had not lost ^{14}C-AIB survived the conversion to protoplasts. The results also show that the protoplasts formed were extremely stable since they maintained their high level of ^{14}C-AIB for over 1 hr.

That the yield of protoplasts was in fact related to the length of time the cells were suspended in sucrose was indicated by an experiment in which this suspension period was considerably reduced. In this experiment, the same procedure for protoplast formation as described in Fig. 7 was applied, except that the time in sucrose was limited to 0.5 instead of 1.5 hr. This length of time is sufficient to convert over 98% of whole cells to mureinoplasts (Table 2). The results (Table 4) show that in this experiment 55% of the mureinoplasts survived conversion to protoplasts, whereas in the previous experiment only 25% had survived. Again, there was the close correlation between ^{14}C-AIB retention and the number of mureinoplasts converted to protoplasts. Thus, as in the previous experiment, those protoplasts which were formed contained the same amount of ^{14}C-AIB as was present in the original whole cells.

Capacity of mureinoplasts and protoplasts to transport ^{14}C-AIB. A suspension of whole cells was converted first to mureinoplasts and then to protoplasts. At each stage, equal samples of the suspension were tested for the capacity of the cell forms suspended in them to take up ^{14}C-AIB. The results (Fig. 8) show that suspensions of mureinoplasts and protoplasts accumulated 47 and 30%, respectively, of the ^{14}C-AIB taken up by the suspension of whole cells from which they were derived.

A number of experiments similar to the one shown in Fig. 8, demonstrating uptake, and of the type illustrated in Fig. 7, showing retention at the various stages in protoplast formation, were performed. In Fig. 9, uptake and retention are compared in typical experiments in which conditions for the formation of mureinoplasts and protoplasts were maintained as nearly identical as possible. It is evident that within the limits of experimental error there is a direct correspondence between uptake and retention capacity at each stage in protoplast formation.

It has been shown that the per cent of ^{14}C-AIB retained indicates the percentage of cells which have survived conversion to protoplasts (Fig. 7 and Table 4). If one also assumes that the per cent retention of ^{14}C-AIB at the mureinoplast stage indicates the per cent of the whole cells which have been converted to undamaged mureinoplasts, one can correct the amount of uptake or retention of ^{14}C-AIB at each stage of protoplast formation for the proportion of intact cell forms present. Applying this correction to the

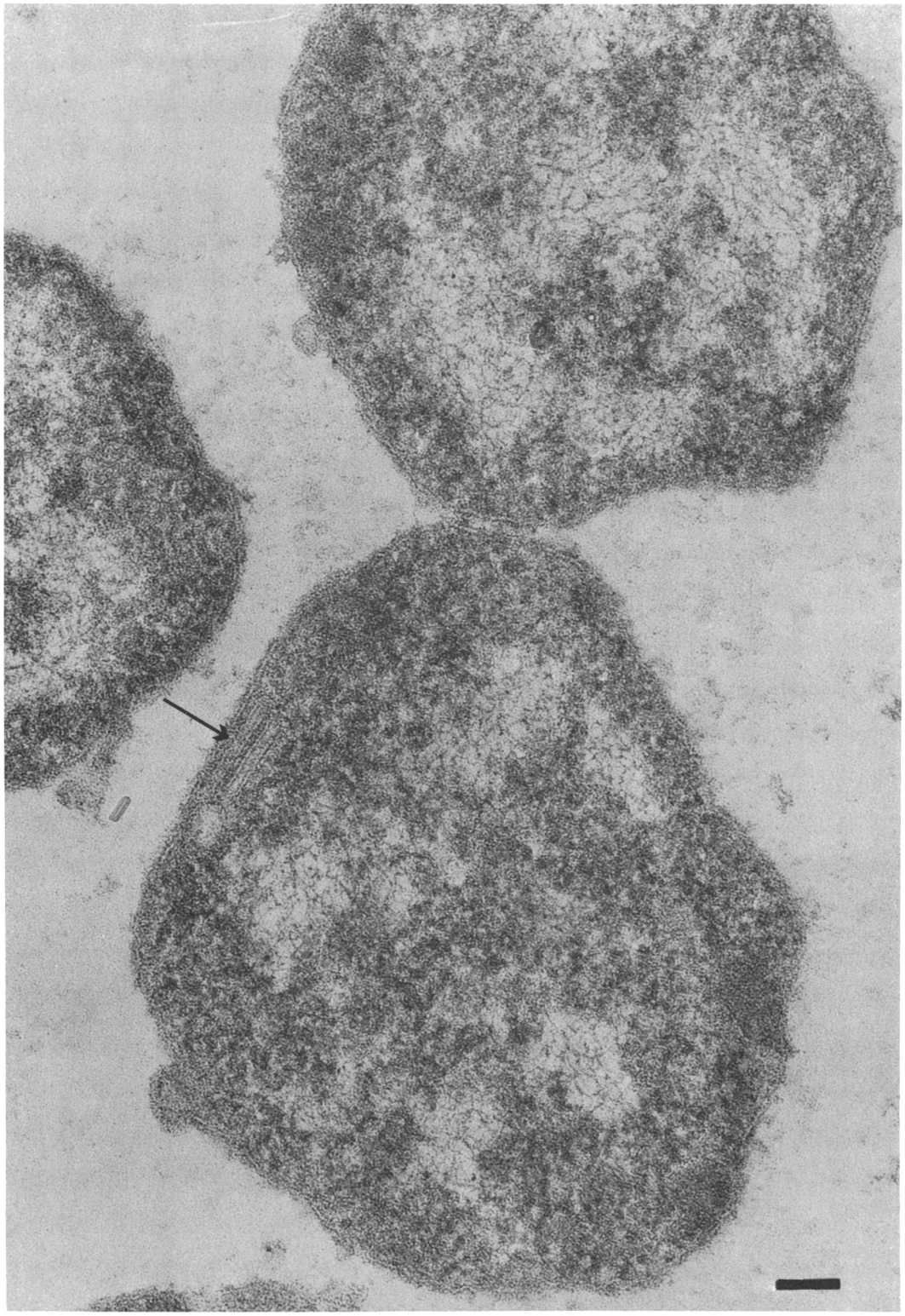

FIG. 6. *Electron micrograph of a thin section of protoplasts 1 hr after the addition of lysozyme.* [*Note the absence of the double track wall structure and the presence of a mesosome* (*arrow*).]

TABLE 2. *Per cent of cells with residual cell wall material remaining after suspension in 0.5 M sucrose as determined by analyses of thin sections in the electron microscope*[a]

Time in 0.5 M sucrose (hr)	Mureinoplasts (no visible outer layer)	Partial wall visible	Complete wall present
0.5	98	1.5	0.75
1.5	100	0.00	0.00

[a] More than 200 cells per sample were analyzed.

TABLE 3. *Removal of hexosamine from mureinoplasts by lysozyme during protoplast formation*[a]

Incubation period (min)	Hexosamine retained (μg)	
	No lysozyme	Plus lysozyme
0	80	80
5		20
30	80	19

[a] Suspensions of mureinoplasts were incubated with and without lysozyme according to the procedure for protoplast formation. The resulting cell forms were centrifuged from the incubation medium and resuspended in a volume of complete salt solution equal to the volume of the original suspension. The hexosamine remaining in equal samples of the resulting suspensions was determined. The residual hexosamine in the protoplasts formed after treatment with lysozyme represents approximately 0.07% of the total envelope hexosamine, based on the value obtained from whole cells washed with 0.5 M NaCl.

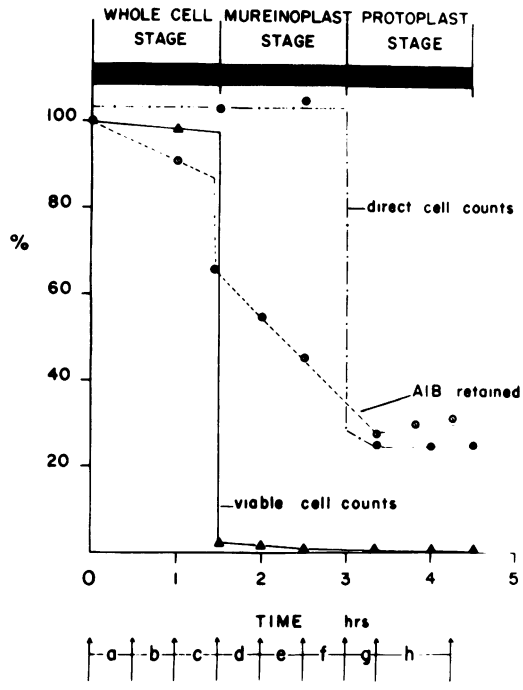

FIG. 7. *Direct counts, viable counts, and ^{14}C-AIB retained during the preparation of protoplasts. The sequence of manipulations was as follows: (a) cells centrifuged from medium and resuspended in 0.5 M NaCl; (b) suspension centrifuged and cells resuspended in 0.5 M NaCl; (c) suspension centrifuged and cells resuspended in 0.5 M sucrose; (d) suspension incubated for 30 min at 25 C; (e) suspension centrifuged and cells resuspended in 0.5 M sucrose; (f) suspension centrifuged and cells resuspended in protoplasting medium; (g) incubation time in protoplasting medium; and (h) period of further incubation of protoplast suspension.*

uptake results in Fig. 8, one arrives at the results in Fig. 10, which show that protoplasts and undamaged mureinoplasts accumulated ^{14}C-AIB to essentially full capacity, i.e., to the same extent as an equal number of whole cells.

Na^+ requirement for transport by protoplasts. The specific requirement of this marine pseudomonad for Na^+ to transport ^{14}C-AIB and other compounds into the cell has been well documented (13, 37). To determine whether this requirement for Na^+ is at the level of the cytoplasmic membrane, two types of experiment were performed. In the first, only the NaCl concentration in the protoplasting medium was varied. The capacity of the protoplasts formed in this medium to take up ^{14}C-AIB was then tested. The results (Fig. 11, curve 2) show that the requirement of the protoplasts for NaCl for optimal rate of uptake of ^{14}C-AIB was about 0.3 M. Examination of the protoplast suspensions by phase-contrast microscopy showed, however, that below 0.2 M NaCl most of the protoplasts had lysed. When LiCl was added to the protoplasting medium at concentrations such that the total of the NaCl and LiCl concentrations were maintained constant at 0.5 M, the requirement for Na^+, though still absolute, was greatly reduced (Fig. 11). Examination of the latter suspensions by phase-contrast microscopy revealed that in all of the combinations of NaCl and LiCl tested the protoplasts remained intact. The whole cells from which the protoplasts were derived were also tested for their requirements for Na^+ for transport in the presence and absence of Li^+. The results (Fig. 11) show that the requirements of whole cells and protoplasts for Na^+ both in the presence and absence of Li^+ were essentially the same. In the case of the whole cells, however, the cells remained intact below 0.2 M NaCl in the absence of LiCl.

DISCUSSION

When whole cells of this marine pseudomonad are suspended in either 0.5 M NaCl or 0.05 M

TABLE 4. *Relationship between percentage of cells obtained during the various stages and ^{14}C-AIB retention*[a]

Experimental conditions	Cells[b]	^{14}C-AIB[c] (% retained)
	%	
After three washes in 0.5 M NaCl	100	88
After 0.5 hr in 0.5 M sucrose (mureinoplasts)	100	68
One hour after conversion to protoplasts	55	59

[a] The suspension period in 0.5 M sucrose during mureinoplast formation was limited to 0.5 hr.

[b] The term cells refers to either whole cells, mureinoplasts, or protoplasts, depending on the stage of the preparation. The percentage of cells represents the number of each form surviving at each stage. The figures were determined by direct count and are expressed as a percentage of the number of whole cells present in the same volume of suspension initially.

[c] The percentage of ^{14}C-AIB retained represents the amount of radioactivity retained by the cell forms at each stage when equal portions of the suspensions were filtered by using membrane filters (Millipore Corp.).

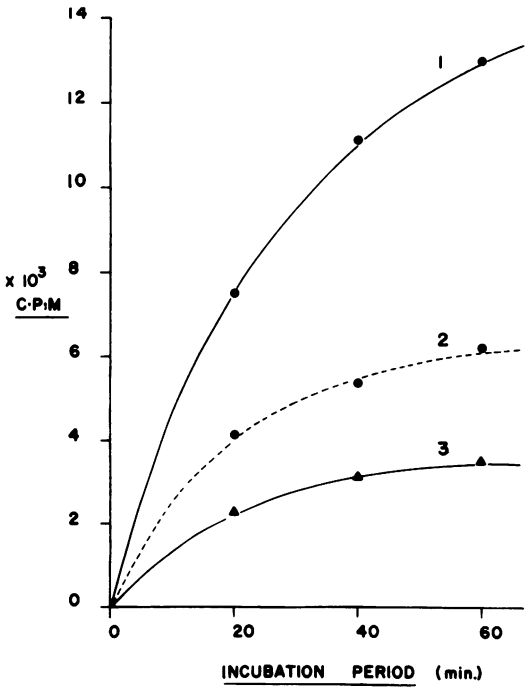

FIG. 8. *Uptake of ^{14}C-AIB by the cell forms present at the various stages in the conversion of cells to protoplasts. Curve 1, whole-cell stage; curve 2, mureinoplast stage; curve 3, protoplast stage.*

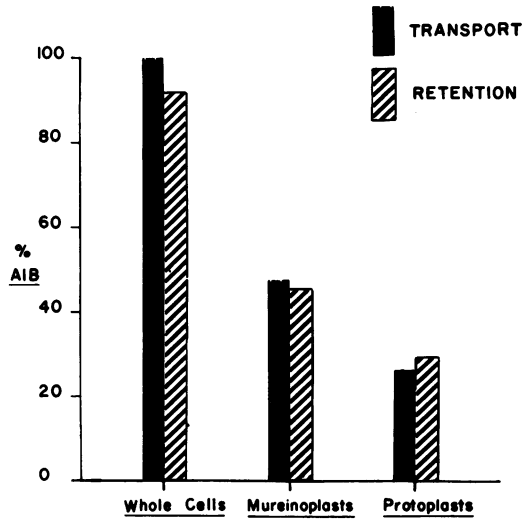

FIG. 9. *Comparison of capacity of cells in various stages in their conversion to protoplasts to take up (transport) and to retain ^{14}C-AIB after 1 hr of incubation.*

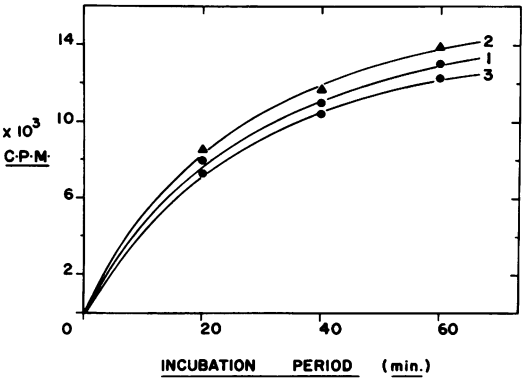

FIG. 10. *Uptake of ^{14}C-AIB by whole cells, mureinoplasts, and protoplasts after correction for the number of undamaged cell forms present in each suspension. Curve 1, whole cells; curve 2, mureinoplasts; curve 3, protoplasts.*

$MgSO_4$ at the cell density used in these studies, no 260 nm-absorbing material can be detected in the suspending solution (22). As the results reported here show, if the outer layers of the cell wall are removed and mureinoplasts are formed, these modified cells lose copious amounts of 260 nm-absorbing material if suspended in either of these salt solutions but not in a solution containing NaCl, $MgSO_4$, and KCl in combination. It may thus be concluded that, to maintain the integrity of the cytoplasmic membrane in mureinoplasts, a balanced salt solution is required. NaCl or $MgSO_4$, when present in the solution

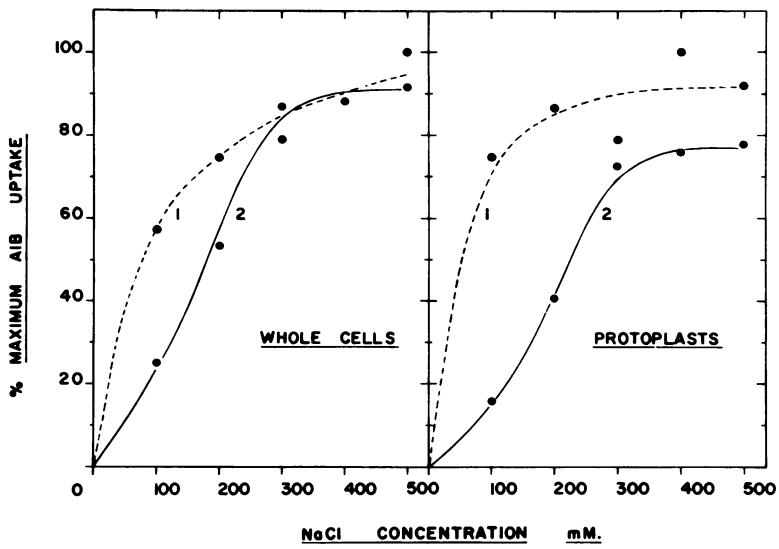

FIG. 11. *Effect of Na^+ in the presence and absence of Li^+ on the uptake of ^{14}C-AIB by cells and protoplasts of the marine pseudomonad B-16 after 15 min of incubation. Curve 1, response to Na^+ when the total of NaCl and LiCl in the suspension was maintained constant at 0.5 M; curve 2, response to Na^+ when NaCl was added in the absence of LiCl.*

alone at appropriate concentrations, apparently increases the porosity of the cytoplasmic membrane sufficiently to permit the release of material from the cells. The fact that this occurs after the removal of the outer layers of the cell wall but not before suggests that the outer layers of the cell wall stabilize the membrane and prevent its distortion by excessive concentrations of individual salts. Although, the manner in which this stabilization is brought about is a matter for speculation at the moment, the cell envelope of this organism is known to be a multilayered structure (C. Forsberg, J. W. Costerton, and R. A. MacLeod, *unpublished data*), each layer of which is very probably cross-linked to each adjacent layer by a multiplicity of bonds. Such bonding could be expected to reduce the degree of distortion which would be produced if the unprotected membrane components interacted directly with nonphysiological concentrations of salts.

The presence of sucrose in a balanced salt solution caused mureinoplasts to leak both 260 nm-absorbing material and ^{14}C-AIB from the cells. Thus, sucrose appeared also to have a capacity under appropriate conditions to increase the porosity of the cytoplasmic membrane of this organism. The mechanism of this effect is obscure at this time.

Since the salt solution in which the protoplasts were suspended contained the salts at concentrations similar to those required for optimal growth by the whole cells, it might at first appear that the protoplasts were no more fragile than the cells from which they were derived. This, however, is not the case. Studies have shown that the combination of salts used in the complete salt mixture are present at a concentration which is more than sufficient to maintain the whole cells intact. Removal of the cell wall layers increases the concentration of the balanced salt mixture needed to maintain the protoplasts intact, but the concentration required does not exceed that present in the complete salt solution (M. K. Rayman and R. A. MacLeod, *unpublished data*). Since NaCl is known to equilibrate across the cytoplasmic membrane of this organism (33), the capacity of the balanced salt solution to maintain the protoplasts intact must be due not to osmotic action but to an interaction of the salts with components of the membrane.

Evidence has been presented to indicate that the protoplasts formed from cells of the marine pseudomonad are essentially free from wall material. Several reports on "membrane" transport in gram-negative bacteria have confused readers by the broad application of the term "membrane" to the intact or partially intact envelope. The term cytoplasmic membrane, as employed here, refers to that structural entity which appears in thin section as a "double track" measuring approximately 7.5 nm in width and located next to the cytoplasm, a definition similar to that proposed by Salton (30).

When cells which had been washed with 0.5 M

NaCl were suspended in 0.5 M sucrose, they lost their viability immediately and essentially completely. This loss of viability coincided with the loss of the outer double-track layer of the organism. There are a number of possible reasons for this loss of viability. One is that the removal of the outer double-track layer could lead to the loss of essential enzymes from the periplasmic space (17).

Mureinoplasts were formed when cells washed with 0.5 M NaCl were suspended in 0.5 M sucrose. Examination of these forms by phase and electron microscopy revealed no obvious damage to the cytoplasmic membrane. Furthermore, unless the time of suspension in the sucrose solution was prolonged, there was no 260 nm-absorbing material released. The ^{14}C-AIB retention studies revealed, however, that more subtle changes were occurring during the period of suspension in the sucrose solution. These changes led to the release of ^{14}C-AIB from a part of the cell population. Only a proportion of the mureinoplasts formed protoplasts, and the protoplasts formed contained as much ^{14}C-AIB as the original untreated cells. Thus, those mureinoplasts which had undergone sufficient damage to lose ^{14}C-AIB did not survive conversion to protoplasts. Since the yield of protoplasts increased as the time of suspension of the cells in the sucrose solution was reduced, one can conclude that the longer the mureinoplasts were suspended in sucrose the greater became the number of the latter which were damaged.

The results obtained here show that the protoplasts formed have the same capacity to transport ^{14}C-AIB as the whole cells. Thus, the complete transport system is located in or upon the cytoplasmic membrane of this organism. No evidence was obtained, direct or indirect, for wall participation in the transport process. This is contrary to the conclusion reached by Brown et al. (7), on the basis of indirect evidence obtained with another marine pseudomonad, that the wall apparently played a role in the transport of solutes into these cells.

Previous studies with whole cells of this organism have been interpreted as indicating that Na^+ has two functions in these cells. One function, which is highly specific for Na^+, is involved in the uptake of ^{14}C-AIB (14, 37), and the other, which can be carried out at least partially by Li^+, prevents the release of the accumulated compound by the cells (37). Since protoplasts and whole cells had the same quantitative requirements for Na^+ for uptake of ^{14}C-AIB and since Li^+ exerted the same degree of sparing action on the Na^+ requirement, it can be concluded that both functions of Na^+ are carried out at the level of the cytoplasmic membrane.

ACKNOWLEDGMENTS

We thank J. M. Weilandt for excellent technical assistance throughout this work.

This investigation was supported by a grant from the National Research Council of Canada.

LITERATURE CITED

1. Albers, R. W. 1967. Biochemical aspects of active transport. Annu. Rev. Biochem. 36:727–756.
2. Anraku, Y. 1967. The reduction and restoration of galactose transport in osmotically shocked cells of *Escherichia coli*. J. Biol. Chem. 242:793–800.
3. Anraku, Y. 1968. Transport of sugars and amino acids in bacteria. III. Studies on the restoration of active transport. J. Biol. Chem. 243:3128–3135.
4. Bibb, W. R., and W. R. Straughn. 1964. Inducible transport system for citrulline in *Streptococcus faecalis*. J. Bacteriol. 87:815–822.
5. Britten, R. J., and F. T. McClure. 1962. The amino acid pool in *Escherichia coli*. Bacteriol. Rev. 26:292–335.
6. Brock, T. D., and G. Moo-Penn. 1962. An amino acid transport system in *Streptococcus faecium*. Arch. Biochem. Biophys. 98:183–190.
7. Brown, A. D., D. G. Drummond, and R. J. North. 1962. The peripheral structures of gram-negative bacteria. II. Membranes of bacilli and spheroplasts of a marine pseudomonad. Biochim. Biophys. Acta 58:514–531.
8. Buckmire, F. L. A., and R. A. MacLeod. 1965. Nutrition and metabolism of marine bacteria. XIV. On the mechanism of lysis of a marine bacterium. Can. J. Microbiol. 11:677–691
9. Cohen, G. N., and J. Monod. 1957. Bacterial permeases. Bacteriol. Rev. 21:169–194.
10. Costerton, J. W., C. Forsberg, T. I. Matula, F. L. A. Buckmire, and R. A. MacLeod. 1967. Nutrition and metabolism of marine bacteria. XVI. Formation of protoplasts, spheroplasts, and related forms from a gram-negative marine bacterium. J. Bacteriol. 94:1764–1777.
11. DeVoe, I. W., and E. L. Oginsky. 1969. Antagonistic effect of monovalent cations in maintenance of cellular integrity of a marine bacterium. J. Bacteriol. 98:1355–1367.
12. DeVoe, I. W., and E. L. Oginsky. 1969. Cation interactions and biochemical composition of the cell envelope of a marine bacterium. J. Bacteriol. 98:1368–1377.
13. Drapeau, G. R., and R. A. MacLeod. 1963. Na^+-dependent active transport of α-aminoisobutyric acid into cells of a marine pseudomonad. Biochem. Biophys. Res. Commun. 12:111–115.
14. Drapeau, G. R., T. I. Matula, and R. A. MacLeod. 1966. Nutrition and metabolism of marine bacteria. XV. Relation of Na^+-activated transport to the Na^+ requirement of a marine pseudomonad for growth. J. Bacteriol. 92:63–71.
15. Fox, C. F., and E. P. Kennedy. 1965. Specific labelling and partial purification of the M protein, a component of the β-galactoside transport system. Proc. Nat. Acad. Sci. U.S.A. 54:891–899.
16. Gale, E. F. 1947. The assimilation of amino acids by bacteria. I. The passage of certain amino acids across the cell wall and their concentration in the internal environment of *Streptococcus faecalis*. J. Gen. Microbiol. 1:53–76.
17. Heppel, L. A. 1967. Selective release of enzymes from bacteria. Science (Washington) 156:1451–1455.
18. Kaback, H. R., and E. R. Stadtman. 1966. Proline uptake by an isolated membrane preparation of *Escherichia coli*. Proc. Nat. Acad. Sci. U.S.A. 55:920–927.
19. Kepes, A., and G. N. Cohen. 1962. Permeation, p. 179–221. *In* I. C. Gunsalus and R. Y. Stanier (ed.), The bacteria, vol. 4. Academic Press Inc., New York.
20. Koch, A. L. 1964. The role of permease in transport. Biochim. Biophys. Acta 79:177–200.
21. MacLeod, R. A. 1965. The question of the existence of specific marine bacteria. Bacteriol. Rev. 29:9–23.

22. MacLeod, R. A., and T. I. Matula. 1961. Solute requirements for preventing lysis of some marine bacteria. Nature (London) **192**:1209–1210.
23. Mora, J., and E. E. Snell. 1963. The uptake of amino acids by cells and protoplasts of *S. faecalis*. Biochemistry **2**:136–141.
24. Osborn, M. J., W. L. McLellan, and B. L. Horecker. 1961. Galactose transport in *Escherichia coli*. III. The effect of 2,4-dinitrophenol on entry and accumulation. J. Biol. Chem. **236**:2585–2589.
25. Pardee, A. B. 1968. Membrane transport proteins. Science (Washington) **162**:632–637.
26. Piperno, J. R., and D. L. Oxender. 1968. Amino acid transport systems in *Escherichia coli* K12. J. Biol. Chem. **243**: 5914–5920.
27. Rickenberg, H. V., G. N. Cohen, G. Buttin, and J. Monod. 1956. La galactoside permease d'*Escherichia coli*. Ann. Inst. Pasteur **91**:829–857.
28. Rothfield, L., and A. Finkelstein. 1968. Membrane biochemistry. Annu. Rev. Biochem. **37**:463–496.
29. Ryter, A., and E. Kellenberger. 1958. L'inclusion au polyester pour l'ultramicrotomie. J. Ultrastruct. Res. **2**:200–214.
30. Salton, M. R. J. 1967. Structure and function of bacterial cell membranes. Annu. Rev. Microbiol. **21**:417–442.
31. Scarborough, G. A., M. K. Rumley, and E. P. Kennedy. 1968. The function of adenosine-5′-triphosphate in the lactose transport system of *E. coli*. Proc. Nat. Acad. Sci. U.S.A. **60**:951–958.
32. Seitz, E. W., and R. M. Hochster. 1965. Active transport of L-valine by *Streptococcus diacetilactis*. J. Dairy Sci. **48**: 1282–1286.
33. Takacs, F. P., T. I. Matula, and R. A. MacLeod. 1964. Nutrition and metabolism of marine bacteria. XIII. Intracellular concentrations of sodium and potassium ions in a marine pseudomonad. J. Bacteriol. **87**:510–518.
34. Weibe, W. J., and G. B. Chapman. 1968. Fine structure of selected marine pseudomonads and achromobacters. J. Bacteriol. **95**:1862–1873.
35. Weidel, W., and H. Pelzer. 1964. Bagshaped macromolecules —a new outlook on bacterial cell walls. Advan. Enzymol. **26**:193–232.
36. Winkler, H. H., and T. H. Wilson. 1966. The role of energy coupling in the transport of galactosides by *Escherichia coli*. J. Biol. Chem. **241**:2200–2211.
37. Wong, P. T. S., J. Thompson, and R. A. MacLeod. 1969. Nutrition and metabolism of marine bacteria. XVII. Ion dependent retention of an intracellular solute and its relation to Na^+ dependent transport in a marine pseudomonad. J. Biol. Chem. **244**:1016–1025.