Periplasmic Space in Salmonella typhimurium and Escherichia coli*

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JEFFRY B. STOCK, BARBARA RAUCH, AND SAUL ROSEMAN

From the McCollum-Pratt Institute and the Department of Biology, The Johns Hopkins University, Baltimore, Maryland 21218

The volume of the periplasmic space in Escherichia coli and Salmonella typhimurium cells was measured. This space, in cells grown and collected under conditions routinely used in work with these bacteria, was shown to comprise from 20 to 40% of the total cell volume. Further studies were conducted to determine the osmotic relationships between the periplasm, the external milieu, and the cytoplasm. Results showed that there is a Donnan equilibrium between the periplasm, the extracellular fluid, and the cytoplasm and that the periplasm and cytoplasm are isoosmotic. In minimal salts medium, the osmotic strength of the cell interior was estimated to be approximately 300 mosM, with a net pressure of approximately 3.5 atm being applied to the cell wall.

A corollary of these findings was that an electrical potential exists across the outer membrane. This potential was measured by determining the distributions of Na+ and Cl− between the periplasm and the cell exterior. The potential varied with the ionic strength of the medium; for cells in minimal salts medium it was approximately 30 mV, negative inside.

Salmonella typhimurium and Escherichia coli have been extensively used as model systems for biochemical studies relating to almost every aspect of cell function. In these investigations they have invariably been considered as single compartments with their cytoplasmic membranes surrounded by cell envelopes composed of two layers: outer lipopolysaccharide membrane, cell wall, and inner plasma membrane (1, 2). This view was not greatly altered by the discovery that several water-soluble proteins were located between the cell wall and the plasma membrane (for review see Ref. 3). The location of these proteins was designated the periplasm (4). The space they occupy, the periplasmic space, may intuitively be regarded as a cell compartment. There have been no reports, however, which define the volume of this hypothetical compartment, its osmotic properties, and whether these parameters change with changes in nutritional state, extracellular osmolarity, etc.

As shown in this report, the periplasm is a second and important compartment of the whole cell, containing at least 20% of the total cell water, and the outer membrane behaves as a Donnan membrane. A preliminary report has been presented (5).

EXPERIMENTAL PROCEDURES

Materials

[U-14C]Sucrose† (350 to 500 Ci/mol), [1-14C]methyl β-D-thiogalactopyranoside (2 to 10 mCi/mol), [D-14C]mannitol (45 to 55 Ci/mol), [1-14C]-N-acetyl-D-glucosamine (30 to 50 Ci/mol), pyrogen-free inulin-[1-14C]carboxyl (1 to 3 mCi/g), inulin-[3H]methoxy (50 to 150 mCi/g), dextran-[1-14C]carboxyl, molecular weight 16,000 (4.7 mCi/g), L-[1-14C]glutamic acid (200 Ci/mol), [1-14C]water (25 mCi/g), [35S]Na+ (carrier-free), and [35S]Cl− (1 to 5 mCi/g) were from New England Nuclear; Triton X-100, 2,5-diphenyloxazole (PPO), dimethyl 1,4-dil2-(5-phenyloxazolyl)benzene (dimethyl-POPOP), and Soluene 100 were from the Packard Instrument Co., La Grange, Ill.; sodium poly-L-glutamate, molecular weight 46,700, was from Sigma; methyl β-D-thiogalactopyranoside was from Calbiochem; glutaraldehyde was from Ladd Research Industries, Burlington, Vt. All other chemicals were reagent grade from standard commercial sources. Siliclad is a product of the Clay Adams division of Becton-Dickinson, Rutherford, N. J.

Bacterial Strains

Salmonella typhimurium LT2 and S. typhimurium LT2 Hartman were from Dr. P. E. Hartman, Johns Hopkins University. These two wild type strains gave identical results. The rough

† Commercial [14C]sucrose was, at best, approximately 98% pure as determined by paper electrophoresis in 1% borate, pH 9.5, for 30 min at 3 kV, or descending paper chromatography in 1-butanol/pyridine/water, 6:4:3 by volume. Much of the contaminating material was glucose. Since Salmonella typhimurium and Escherichia coli are unable to absorb sucrose, but can rapidly accumulate glucose, it was necessary to purify the [14C]sucrose. After paper electrophoresis in 1.0% borate, [14C]sucrose was eluted with water, and the eluent was deionized with mixed bed resin (AG 50-H+ and AG 3-OH−). Just before use with bacterial cells, the labeled sucrose solution was incubated for 30 min with an aliquot of the same bacterial suspension. The cells were then removed, first by centrifugation (10 min at 19,000 × g), then by filtration (0.22 μM Millipore filters). Over 99% of the resulting radiolabeled material was found to be [14C]sucrose and was susceptible to hydrolysis by invertase.

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strains, SL3555 (his[rfb]-386galE742) and SL756(galE), both derivatives of S. typhimurium LT2, were also from Dr. P. E. Hartman. Dr. J. C. Cardoso, Johns Hopkins University, contributed strain SL349 (topB22454,galP247). The Escherichia coli strains, K12 and ML308-255, were from the collection of Dr. W. Kudnig, Johns Hopkins University.

Methods

Growth and Collection of Cells

Cells were usually grown in Medium 63 (8) supplemented with the carbon source and other nutrients (as indicated) but without iron. Cultures were maintained at 37° in a New Brunswick shaker incubator set at 200 rpm or in a water bath with aeration (0.5 liter of air/min). A few hours before cells were required for experiments, overnight cultures were diluted 1:10 into growth medium and the increasing turbidity was monitored at 500 nm in a Gilford spectrophotometer. Cells were harvested during the exponential phase of growth (A, 0.5 to 0.9) by a 10-min centrifugation at 10,000 x g in a Sorvall RC2B centrifuge. To wash the cells, the pellets were resuspended in wash medium (generally Medium 63), centrifuged for 5 min at 10,000 x g, and resuspended in a volume of wash medium equivalent to one-hundredth the culture volume before harvesting. This procedure gave suspensions containing 10 to 20 mg, dry weight, of cells/ml. Unless otherwise indicated, all steps (centrifugation, storage, etc.) were performed at room temperature.

Measurement of Solute Distributions

The cytoplasmic, periplasmic, and total cell volumes were all derived from the distributions of solutes in cell suspensions. The rationale is illustrated in Fig. 1. Inulin, because it is large, cannot penetrate the outer membrane and is located only in the extracellular fluid. Sucrose, without the aid of a permease, cannot cross the plasma membrane and is located only in the periplasm and extracellular fluid. Water moves freely throughout the cell; it is located in the cytoplasm, periplasm, and extracellular fluid. As indicated in the figure, determination of the volumes occupied by these compounds gives, by difference, the total, cytoplasmic, and periplasmic volumes of the cells in a suspension.

Solute distributions were measured by three procedures, all of which involved the use of radiolabeled compounds. They differed in the methods used to separate cells in suspension from the bulk of their surrounding medium.

Centrifugation Method—Two 2-ml aliquots of a cell suspension containing from 2 to 10 mg, dry weight, of cells/ml were used for each measurement. A pair of radiolabeled compounds was added to one suspension but not the other. The labels were usually 3H]water and either [14C]sucrose or inulin-[14C]carboxyl. Radioactive compounds were added so that the higher energy isotope (usually 14C) gave at least 1000 cpm/0.1 ml of suspension, and the lower energy isotope (always 3H) contributed 10 to 100 times more counts to the same sample volume.

Unless indicated otherwise, within 1 min of adding radiolabel, both 2-ml cell suspensions, labeled and unlabeled, were centrifuged in 5-ml polypropylene centrifuge tubes for 5 min at 10,000 x g (SS-34 rotor). The supernatant fluids were removed and 0.10 ml of the labeled supernatant was transferred to the unlabeled pellet. The pellets were each resuspended in 1.0 ml of Medium 63 and transferred to liquid scintillation counting vials each containing 15 ml of scintillation fluid (from a mix of 16.5 g of 2,5-diphenyloxazole, 0.375 g of dimethyl-1,4-di-(2-(5-phenyloxazolyl))benzene, 250 ml of Triton X-100, and 667 ml of toluene (7)). Radioactivity was then assayed in a Packard liquid scintillation spectrometer.

This procedure gave two values, one corresponding to the counts per min of radiolabel in the pellet (C), the other to the counts per min of labeled supernatant (C). It was convenient to express the amount of labeled solute in a cell pellet as a volume. For a particular solute (S) this was termed V, which was computed (in milliliters) using the formula:

\[ V_S = \frac{C}{C} \times \frac{1}{10} \]

For a centrifuged cell suspension, V was equal to the volume of supernatant which contained the same amount of solute (S) as the pellet. With chemicals which were not concentrated by the cells such as [3H]water, [14C]sucrose, or unlabeled [14C]carboxyl, the differences between V values may be used to compute cell volumes as illustrated in Fig. 1.

Oil Filtration Method—In this procedure, cells suspended in solutions of labeled solutes were filtered through Siliclad-treated glass fiber filters. The technique permitted relatively rapid (15 to 30 s) filtration, gave minimal blank values (extracellular liquid adsorbed to the filters), and prevented evaporation of the [3H]water used in many experiments.

Cell suspensions with and without labeled solutes were prepared as described under "Centrifugation Method" except that approximately 10 times more label was added. The filtration apparatus (for a detailed description see Ref. 8) consisted of a vacuum manifold designed to hold 30 Siliclad-treated filters. A chamber above the filters was filled with oil (paraffin oil, Fisher Scientific Co.) before 0.10-ml aliquots from the radionuclide labeled suspension were filtered. Each filter was then transferred from under the oil to a liquid scintillation vial containing 1.0 ml of Soluene 100 and incubated at 30° for 3 h during which time over 90% of the radiolabel was solubilized. Scintillation fluid (15 ml of the mixture described above) was added to each of the samples which were then left overnight at room temperature before being assayed for radioactivity in a Packard liquid scintillation spectrometer. The resulting numbers were designated C (see below).

Several independent tests showed that the oil filtration method gave valid results. If solutions of radiolabeled solute (without cells) were passed through filters the amounts of label retained were equal only to those contained in the small volumes of solution adhering to the filters.1 If cells were grown in the presence of L-[14C]histidine they could be shown to be quantitatively retained by Siliclad-treated filters. Furthermore, data obtained by oil filtration were consistent with results derived from other procedures (see "Results").

Dilution-Filtration Method—A cell suspension containing radiolabeled solutes was prepared as described under "Oil Filtration Method," but with only one label, the higher energy isotope.2 Aliquots of 0.10 ml were transferred from this suspension into 10 ml

1 Reeve Angel glass fiber filters (No. 984H, custom cut to 5/8 inch diameter) were added to 1 liter of 2% Siliclad in a 1-liter glass beaker. The beaker was placed in a water-filled Bronson Sonifier and sonicated for 1 min. The Siliclad solution was then replaced with 1 liter of water. After a 10-min period of sonication the filters were removed and dried overnight in an oven at 80°.

2 Most commercial preparations of radiolabeled inulin or dextran adsorbed to these filters. Pyrogen-free inulin-[14C]carboxyl from New England Nuclear was the only labeled polysaccharide tested, which was not adsorbed.

3 In some experiments [3H]water was present as a second label. If so, the labeled water was removed by drying the filters before they were placed in scintillation fluid.
of wash medium (see "Growth and Collection of Cells"), and the resulting 1:100 diluted samples were immediately filtered through 2.5-cm Siliclad-treated filters. Approximately 5 s elapsed between dilution of a 0.10-ml aliquot and its complete filtration. Each filter was placed in a vial containing 15 ml of scintillation fluid (see above), and radioactivity was assayed in a Packard spectrometer. The radioactivity per unit volume of original suspension was determined by adding 0.010 ml of undiluted cell suspension to a filter, which was then added to a vial containing 15 ml of scintillation fluid, and assaying for radioactivity. The data derived from these procedures were used in the same way as analogous information obtained from oil filtration (see Equation 2).

As determined by dilution-filtration, the amount of labeled solute within the cells of a 0.10-ml suspension aliquot was taken as the difference between the total label retained by a filter and the background due to adsorption of extracellular fluid. Cells labeled by growth in the presence of L-[14C]histidine were used to show that the radioactivity on filters after diluting and filtering 0.10-ml aliquots from cell suspensions containing inulin-[14C]carboxy. It was equivalent to 0.20 μl of undiluted suspension, approximately 20 μl of the 10 ml actually filtered. Similarly, if radiolabeled solutions without cells were diluted and passed through filters, the label adsorbed was equal to the amount in 0.20 μl of undiluted solution. This was true for all of the radioactive solutes used except the polysaccharides.*

Determination of Intracellular Osmotic Strength by Vapor Phase Equilibrium Method

The method used for determining intracellular osmotic strength was modified after that of Mitchell and Moyle (9). Cells were prepared as described under "Growth and Collection of Cells" except that they were washed with water instead of Medium 63. Aliquots (1.0 ml each) of the resulting suspensions were placed on tared glass cover slips and lyophilized. Each cover slip was then placed over the center well of a Conway microdiffusion dish; solutions of sucrose were added to the outer wells, the dishes were covered, and mineral oil was added to the sealing wells. Dishes were set up in duplicate, each pair containing a different concentration of sucrose. After 1 day of incubation at room temperature, one cover slip from each pair was weighed and placed in the water which had condensed on it. A day later the remaining cover slips were weighed. Since no significant differences were observed between these two sets of results, it was concluded that equilibrium had been attained by the end of the first day.

The osmotic strength (osmolarity) of a solution of volume W may be expressed as X/W where X corresponds to the number of moles of ideal solute (osmoles) which would, if dissolved in W liters, account for the observed osmotic pressure, or any other colligative property, of the solution. In these terms, the osmotic strength of the solution on a cover slip could be represented as X/W with W being the amount of water that had condensed. At equilibrium the osmotic strength of the material on a cover slip should have been equal to the osmotic strength of the sucrose solution below it (9). Empirically, the osmotic strength of a sucrose solution is approximately equal to its molal concentration (sucrose) (10), rather than its molarity. It follows that:

\[
X/W = (\text{sucrose})
\]

Assuming X remained constant over the range of sucrose concentrations, a plot of (sucrose) versus 1/W should have been linear, with slope corresponding to X. The osmotic strength of cells in water was estimated from this slope divided by the water content of cells prior to lyophilization: i.e. \(X/V_{cell} = V_{cell}\) determined from solute distribution measurements (see above).

Electron Microscopy

Two procedures were used to obtain thin sections of bacteria for examination in a Jeol model 100B electron microscope. Glutaraldehyde Fixation - Cells were harvested, washed twice, and resuspended as described under "Growth and Collection of Cells." Wash medium was either Medium 63 or Medium 63 containing 1.0 M sucrose. Glutaraldehyde was added to the suspension at room temperature to a final concentration of 0.5%; the resulting suspension was incubated for 1 h at room temperature and then centrifuged. After again suspending the cells in Medium 63 or Medium 63 containing 1.0 M sucrose, they were put through an ethanol dehydration schedule. This involved centrifugation and suspension in successively increasing concentrations of ethanol in water (2-ml portions of 50, 70, 80, 95%, and absolute ethanol were used). Dehydrated cells were then embedded in plastic and sectioned. All manipulations prior to dehydration were conducted at 0°. Thin sections were stained for 30 min in a 6% solution of uranyl acetate in water and for 3 min in a lead citrate solution (11).

Glutaraldehyde-Osmium Tetroxide Fixation - Cells were harvested, washed twice in 50 mM potassium phosphate, pH 7.3, and resuspended in this buffer (see "Growth and Collection of Cells"). Portions, 20 μl each (approximately 20 mg, dry weight, of cells), were transferred to microfuge tubes containing 0.3 ml of 50 mM

* These were prepared by soaking Reeve Angel glass fiber filters, No. 984H, in a 2.0% solution of Siliclad.
potassium phosphate buffer with 3% glutaraldehyde. The tubes were centrifuged and then left for 2 h before the supernatant solutions were decanted. The resulting pellets were rinsed (without resuspension) three times with 0.3-ml portions of buffer and then left overnight. The next day 0.5-ml portions of a 2% osmium tetroxide solution in 50 mM potassium phosphate, pH 7.3, were added to the fixed pellets. After 2 h this solution was removed, and the pellets were taken through an acetic dehydration schedule consisting of rinses with successively more concentrated acetic solutions (two rinses with 0.3-ml portions of 50%, 70%, 90%, and anhydrous acetic acid). Specimens were then embedded in plastic and sectioned. All manipulations prior to embedding were conducted at 0°. Thin sections were stained for 4 min in a 50% ethanol solution saturated with uranyl acetate and for 10 min in a lead citrate solution.

RESULTS

Solute Distributions

Distributions of inulin, sucrose, and water were measured in suspensions of Salmonella typhimurium. As the data in Table I show, sucrose was able to penetrate a sizable compartment which was not available to inulin. This result was obtained with either the oil filtration or centrifugation method (Table I) and with several different combinations of isotopes (Table II). Thus, according to Fig. 1, these cells were divisible into two compartments, the cytoplasm (V_inulin - V_sucrose) and the periplasm (V_sucrose - V_inulin), each comprising a significant fraction of the whole cell volume (V_water - V_inulin).

The intracellular sucrose pool measured by oil filtration or centrifugation (V_sucrose - V_inulin) was formed within a minute and thereafter remained constant for at least 30 min (data not shown). When sucrose uptake was determined by dilution-filtration, no intracellular pool was observed. Apparently sucrose moved so quickly across the outer membrane that it was lost from the periplasm within 5 s of diluting the extracellular fluid. This conclusion was confirmed by studying the behavior of cells toward another small neutral solute, methyl β-D-thiogalactopyranoside.

TMG is not metabolized by S. typhimurium. It is a substrate for the melibiose permease (12) and cannot be accumulated unless this transport system has been induced by growth on melibiose. Experiments were performed with both induced and uninduced cells. As expected, induced cells accumulated high levels of TMG (Fig. 2). Moreover, each of the three methods (dilution-filtration, centrifugation, and oil filtration) gave the same results. Since under these conditions the amount of TMG in the periplasm was negligible compared to that concentrated within the cytoplasm, the three procedures gave the same measure for cytoplasmic uptake.

Different results were obtained with uninduced cells (see Fig. 3) where, since little TMG entered the cytoplasm, the portion located within the periplasm represented a significant fraction of the total intracellular pool. Under these conditions TMG could be seen to behave similarly to sucrose in that it was lost from the periplasm during dilution-filtration, this difference being the amount of material located within the cytoplasm. Since intracellular TMG as measured by the centrifugation procedure was the sum of both the periplasmic and cytoplasmic pools, the latter method gave larger values for TMG uptake than those obtained by dilution-filtration, the difference being the amount of TMG within the periplasm. In situations where the cytoplasmic pool was relatively large, as when cells were induced for TMG uptake, this difference was, of course, negligible.

Results such as those presented in Fig. 3 indicated that, after correcting for material contained in the cytoplasm, the intracellular level of any low molecular weight neutral solute could be used to determine periplasmic volume. This conclusion was substantiated by measuring the distributions of several small solutes in addition to TMG and sucrose. As shown in Table III, the same periplasmic volumes were obtained with mannitol, N-acetylgalactosamine, sucrose, and TMG. In this experiment, a strain of S. typhimurium defective in its ability to transport sugars was used so as to minimize cytoplasmic uptake.

Electron Microscopy

Fig. 4 is an electron micrograph of an S. typhimurium cell in cross-section. The periplasm is the area bounded on the outside by the cell wall-outer membrane complex and separated from the cytoplasm by the inner cytoplasmic membrane. This figure shows cells fixed in the presence of osmium tetroxide plus glutaraldehyde.

If osmium tetroxide was omitted and cells were fixed in glutaraldehyde alone, it could be shown that their compartmental structure was not destroyed by fixation. That is, inulin, sucrose, and water distributions were not altered by the glutaraldehyde treatment. Furthermore, periplasmic volumes estimated from the structures depicted in electron micrographs of glutaraldehyde-fixed cells agreed with those derived from the distributions of solutes in suspensions of the living organisms. This was true for both normal and plasmolyzed cells (see Fig. 5). Such results provided the link between morphology and function which allowed the conclusion that S. typhimurium LT2 cells are composed of two compartments, cytoplasm and periplasm, each representing a substantial fraction of the bacterial cell volume.

Two Compartment Structure as a General Feature of Salmonella typhimurium and Escherichia coli

To determine whether a particular method of cell preparation was crucial to its final compartmental structure, solute distributions were measured in bacterial suspensions obtained by variants of the standard methods described under "Growth and Collection of Cells." The following aspects of cell preparation were modified: rate of cell proliferation, degree of aeration during growth, nutrients and salts present during growth, phase of growth at harvest, wash medium, and temperature during harvest, wash, or storage. None of the variations tested caused substantial changes in cell compartmentation. For instance, results obtained with S. typhimurium grown and suspended in nutrient broth were similar to those obtained with cells grown, washed, and suspended in Medium 63 (see Table IV).

Several different strains of enteric bacteria were examined to determine whether the compartmental structure observed in S. typhimurium LT2 was a general feature of these types of organisms. As shown in Table IV, a similar division of cellular contents into periplasm and cytoplasm occurs in lipopolysaccharide-defective strains of S. typhimurium (SL3555 and SL756) and in different strains of E. coli (K12 and ML308-255). These results led to the conclusion that S. typhimurium and E. coli are composed of two compartments, periplasm and cytoplasm, and that the periplasm occupies 20 to 40% of the total cell volume under conditions routinely used in work with these bacteria.

The abbreviation used is: TMG, methyl β-D-thiogalactopyranoside.
Cells (Salmonella typhimurium LT2) were grown in Medium 63 containing 0.5% m-lactate. In centrifugation experiments [3H]water (final specific activity, 1.0 x 10⁶ cpm/g) and [14C]sucrose (specific activity, 0.97 x 10⁶ cpm/μmol; final concentration 1.0 mM), or [3H]water (final specific activity, 2.4 x 10⁶ cpm/g) and inulin-[14C]carboxyl (specific activity, 2 x 10⁶ cpm/μmol) were added to suspensions containing 8.0 mg, dry weight, of cells in 2.0 ml of Medium 63. Similar suspensions were prepared for oil filtration except that the final specific activities of [3H]water and [14C]sucrose were increased by a factor of 10. The resulting radiolabeled suspensions were incubated for 30 min at room temperature before being either centrifuged or filtered (0.10-ml aliquots) under oil. In this table the meaning of each value under the heading "Radioactivity" depends on its designation as C₁, C₂, C₃, or C₄ (see Equations 1 and 2). C₁ and C₂ values are averages of results from two centrifugation assays; C₃ values are averages of results from 15 filters, and C₄ values are averages of results from four 0.01-ml samples of labeled suspension. The V₅ values were computed according to Equations 1 and 2 for each determination, and the average of these values is presented. Thus, these V₅ values do not necessarily correspond to the values obtained from the average values for C₁, C₂, C₃, and C₄. The errors presented are for centrifugation data, the deviation of each experimental value from the mean; for oil filtration data, the standard error of each set of measurements.

![Table I](http://www.jbc.org/)

### Table I

**Sucrose, inulin, and water distributions as determined by centrifugation and oil filtration methods**

| Method          | Labels         | Radioactivity | V₅   | V₅[H]water - V₅[14C]CS |
|-----------------|----------------|---------------|------|------------------------|
|                 |                | cpm           | μl   | μl/μg, dry wt. of cells |
| Centrifugation  | [3H]water      | C₁, 50,600 ± 900 | 51.4 ± 2.5 | 19.5 ± 0.4 |
|                 |                | C₂, 98,600 ± 3,200 | 31.9 ± 2.2 | 2.5 ± 0.1 |
|                 | [14C]inulin    | C₃, 2,060 ± 80 | 1.68 ± 0.06 | 0.93 ± 0.04 |
|                 |                | C₄, 6,470 ± 190 | 0.75 ± 0.04 | 2.3 ± 0.1 |
| Oil filtration  | [3H]water      | C₁, 17,000 ± 600 | 53.9 ± 4.5 | 11.0 ± 0.2 |
|                 |                | C₂, 101,000 ± 1,000 | 42.9 ± 4.4 | 1.4 ± 0.0 |
|                 | [14C]sucrose   | C₃, 501 ± 25 | 1.80 ± 0.07 | 0.55 ± 0.05 |
|                 |                | C₄, 6,660 ± 70 | 1.25 ± 0.06 | 1.4 ± 0.1 |

**Total cell volumes measured with different isotope combinations**

Cells (Salmonella typhimurium LT2) were grown in Medium 63 containing 0.5% m-lactate. The culture was harvested, washed, and resuspended as described under "Growth and Collection of Cells." The wash medium was Medium 63 supplemented with 10 mM NaCl. Radiolabeled solute pairs were added to suspensions containing 9.4 mg, dry weight, of cells in 2.0 ml of wash medium. After 30 min at room temperature these suspensions were centrifuged, and solute distributions were determined by the centrifugation method. The radiolabeled solute pairs used were [3H]water or inulin-[14C]methoxy and one of the compounds listed under "Radioactivity." Values for Vₑ问世 were obtained by summing the quantities of V₅water - V₅ and (V₅ - V₅m), for a particular S. Since the total is algebraically equivalent to (V₅water - V₅inulin), its correspondence to Vₑ问世 follows from the scheme outlined in Fig. 1.

**TABLE II**

**Table II**

**Osmotic Relationships between Cytoplasm, Periplasm, and External Medium**

S. typhimurium LT2 cells were exposed to high concentrations of three solutes (Table V): one too large to penetrate the outer membrane (sodium polyglutamate); one able to cross the outer, but not the inner membrane (sucrose); and one able to move freely across both membranes (ethanol). As shown in the table, each of these compounds had a different effect on the cells. A 10% solution of sodium polyglutamate...
by an X in the figure. The "CITMG used in these experiments was dine/water, 6:4:3, and found to be greater than 98% pure. This was bars in the figure. Uptake of TMG was measured as follows: at time 

the dilution-filtration procedure. The results are indicated by trian-

gles (A). In another experiment, ["CITMG was added (final concen-

tration = 1.0 mM) to 2.0 ml of medium containing ["HIwater plus 8 

mg, dry weight, of cells; and after 30 min, 1 ml of the suspension was diluted into 100 ml of Medium 63 containing L"HIwater and 20 mg, dry weight, of cells; and after 30 min the distribution of TMG was determined by the centrifugation method. The result is shown by an X in the figure. The ["CITMG used in these experiments was analyzed by descending paper chromatography in 1-butanol/pyri-

dine/water, 6:4:3, and found to be greater than 98% pure. This was also true of the ["CITMG extracted from a cell pellet. The same results were obtained when the experiments were conducted with Medium 63 containing 10 mM NaCl.

permease. The cells were the same as those used in the experiments 
described in Table I, and the oil filtration data in the table were 
used to estimate the total and cytoplasmic volumes indicated by the 
bars in the figure. Uptake of TMG was measured as follows: at time 

and at the indicated times, TMG distributions were determined by 

the oil filtration (●) and dilution-filtration (○) methods. After 30 

min, 1 ml of the suspension was diluted into 100 ml of Medium 63 

and 10 ml aliquots of this suspension were assayed for intracellular 

["CITMG in the same way as the 10-ml suspensions obtained during 

the dilution-filtration procedure. The results are indicated by trian-
gles (A). In another experiment, ["CITMG was added (final concen-
tration = 1.0 mM) to 2.0 ml of medium containing ["HIwater plus 8 
mg, dry weight, of cells; and after 30 min the distribution of TMG 
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by an X in the figure. The ["CITMG used in these experiments was 
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also true of the ["CITMG extracted from a cell pellet. The same 
results were obtained when the experiments were conducted with 
Medium 63 containing 10 mM NaCl.

TABLE III
Estimations of periplasmic volume from distributions of different solutes in suspensions of Salmonella typhimurium LT2

| Solute (S) | Ratio of (Sout) to (Sint) | Periplasmic fraction of total cell volume |
|------------|------------------------|--------------------------------------|
| [14C]Sucrose | 0.24 ± 0.04 | 0.00 ± 0.01 |
| [14C]CITMG | 0.82 ± 0.09 | 0.43 ± 0.02 |
| [14C]Acetyl-glucosamine | 0.71 ± 0.08 | 0.37 ± 0.02 |
| [14C]Mannitol | 0.85 ± 0.05 | 0.62 ± 0.03 |

caused cells to shrink to less than two-thirds of their normal volume, but did not effect a significant change in the ratio of their periplasmic and cytoplasmic volumes. A 1.0 M solution of sucrose had little effect on total cell volume, but caused swelling of the periplasm and concomitant loss of water from the cytoplasm. Finally, 10% ethanol had no significant effect on either the total cell, periplasmic, or cytoplasmic volumes. These results are explained in terms of the three assumptions enumerated below.

(A) Cytoplasmic Membrane is Flexible and Unable to Support a Pressure Gradient—The concentrated solutions listed in Table V each caused an increase in periplasmic osmotic pressure (\(\Pi_{\text{peri}}\)). When sodium polyglutamate was added to cells the periplasm lost water until its osmotically active contents were sufficiently concentrated to stop the flow. Sucrose and ethanol contributed directly toward an increased \(\Pi_{\text{peri}}\) by rapidly diffusing from the extracellular fluid into the periplasm. Elevated periplasmic pressures caused corresponding changes in the cytoplasm. The cytoplasmic osmotic pressure \(\Pi_{\text{cyto}}\) was increased either by water moving out or by solute moving in. The former occurred with cells in sodium polyglutamate or sucrose, the latter with cells in ethanol. Under all conditions the periplasm and cytoplasm remained isoosmotic, a fact that makes the term intracellular osmotic pressure \(\Pi_{\text{cell}}\) meaningful:

\[
\Pi_{\text{cell}} = \Pi_{\text{peri}} = \Pi_{\text{cyto}}
\]

(B) Cell Wall is Resistant to Pressure Exerted by Periplasm, But Not To That Exerted by External Medium—The osmotic pressure exerted by the cytoplasm of a bacterium may exceed that exerted on the cell by its surrounding fluid (\(\Pi_{\text{ex}}\)). In such circumstances, the cell wall functions to prevent lysis by exerting a pressure \(P_{\text{cell wall}}\) in opposition to that from the cell interior. In Table V, the results obtained with sodium polyglutamate show that if the extracellular osmotic pressure exceeds the intracellular pressure, a cell tends to collapse. The cell wall, like a dialysis bag, is unable to resist compression, and the cell loses water until the osmotically active material within is sufficiently concentrated to make the cell and its environment isoosmotic.

Thus,

\[
\Pi_{\text{cell}} = \Pi_{\text{ex}} + P_{\text{cell wall}}
\]

(C) Aqueous Regions of a Cell Have Colligative Properties Not Appreciably Different from Those of Any Other Solution of Similar Concentration—The osmotic pressure \(\Pi\) that a solution can exert is often expressed in terms of osmotic strength, i.e. the molar concentration \(c\) of an ideal solution which would exert the same osmotic pressure according to the relationship \(\Pi = RTc\) (\(R\) being the gas constant and \(T\) the absolute temperature). The units for osmotic strength are osmoles per liter (osmolarity). From these considerations, if \(V\) is the volume of a cell compartment, the osmotic pressure exerted by its contents is \(RTX/V\), \(X\) being the moles of ideal solute (osmole) in the compartment and \(X/V\) the osmotic strength. Specifically:

\[
\Pi_{\text{peri}} = RTX_{\text{peri}}/V_{\text{peri}}
\]

\[
\Pi_{\text{cyto}} = RTX_{\text{cyto}}/V_{\text{cyto}}
\]
FIG. 4. Cross-section of a Salmonella typhimurium LT2 bacterium. Cells were grown in Medium 63 containing 0.5% pyruvate. They were then processed according to the glutaraldehyde-osmium tetroxide fixation procedure described under "Electron Microscopy."

As long as \( X \) remains constant the osmotic pressure of a cell compartment or a whole cell will vary inversely with its volume. However, a solute will augment the osmotic strength of any compartment it enters. For instance, since sucrose equilibrates across the outer membrane it should contribute to the osmotic strength of the periplasm. If (sucrose) is the molal sucrose concentration in the medium (see "Experimental Procedures")

\[
\Pi_{\text{cell}} = \frac{RT(X_{\text{per}} + X_{\text{cyt}})}{V_{\text{cell}}}
\]

(8)

Changes in cell volume such as those shown in Table V could be used to estimate intracellular osmotic strength. Some examples follow.

With cells in polyglutamate, according to Equation 5: \( \Pi_{\text{cell}} = \Pi_{\text{ex}} + P_{\text{cell wall}} - 0.428 RT + 0 \). When combined with
NORMAL CELLS

PLASMOLYZED CELLS

| Method of estimation | Periplasmic fraction of total cell volume |
|----------------------|-----------------------------------------|
| Electron microscopy   | 0.36                                    |
| Solute distribution measurements | 0.34                                    |
|                       | 0.69                                    |
|                       | 0.77                                    |

Eq. 6, this gives: $X_{\text{Deri}} + X_{\text{cyto}} = 0.428 V_{\text{cell}}$, where $V_{\text{cell}}$ is the cell volume in polyglutamate. If, in Medium 63, the cell volume is $V_{\text{cell}}$, then using the derived value for $X_{\text{Deri}} + X_{\text{cyto}}$ and Eq. 9 gives: $D_{\text{cell}} = 0.428 RT V_{\text{cell}}/V_{\text{cyto}}$. From Table V, the osmotic strength can, therefore, be calculated as 270 mosm in Medium 63. This result agrees well with results obtained by independent methods (see below). Applying this result to Eq. 5 gave a value of 3.3 atm as the net pressure on the cell walls of these bacteria at room temperature in Medium 63.

The intracellular osmotic strength can also be estimated by measuring changes in cell volume in response to extracellular sucrose concentrations. Combining Equations 4, 7, and 9 gives:

$$V_{\text{per}}/V_{\text{cyto}} = X_{\text{per}}/X_{\text{cyto}} + \left( \text{sucrose} \right) V_{\text{per}}/V_{\text{cyto}} \tag{10}$$

Since these experiments involved high concentrations of sucrose, where the solute contributed to the measured periplasmic volume, a correction for the volume of the sucrose itself was required. The corrected equation10

$$V_{\text{per}}/V_{\text{cyto}} = X_{\text{per}}/X_{\text{cyto}} + \left( \text{sucrose} \right) V_{\text{per}}/V_{\text{cyto}} \tag{11}$$

where $V_{\text{per}}$ and $V_{\text{cyto}}$ represent the measured volumes of the respective compartments and [sucrose] is the molar sucrose concentration. Therefore, $V_{\text{per}}/V_{\text{cyto}}$ should vary linearly with $V_{\text{per}}$ [sucrose], and the slope of the line should be $1/X_{\text{cyto}}$. The experimental relationship is shown in Fig. 6 and direct.
The different bacterial strains were grown in nutrient broth or Medium 63 plus the indicated nutrients. Cultures grown on defined medium were harvested, washed with Medium 63, and finally suspended in this medium. The culture in nutrient broth was simply harvested and resuspended in nutrient broth. \[^{1}H\text{water and inulin}^{[14]C}\text{carboxyl or}^{[14]C}\text{sucrose were added to suspensions, and the distributions of these labeled compounds were determined by the centrifugation method. This information was then used in accord with Fig. 1 to estimate the total volume of cells in each suspension and to divide this volume into its cytoplasmic and periplasmic components. These results are expressed as the periplasmic fraction of the total cell volume.}

| Bacterial strain | Nutrients present during growth | Periplasmic fraction of total cell volume |
|------------------|---------------------------------|----------------------------------------|
| *S. typhimurium* LT2 | 0.2% D-glucose | 0.33 |
| *S. typhimurium* LT2 | Nutrient broth | 0.20 |
| SL355 (rfr galE) | 0.2% D-glucose + 0.002% L-histidine | 0.19 |
| SL756 (galE) | 0.2% D-glucose | 0.24 |
| E. coli K12 | 0.2% D-glucose | 0.21 |
| E. coli ML308-225 | 0.2% D-glucose | 0.37 |

Effects of concentrated solutions on total and compartmental volumes of *Salmonella typhimurium* LT2

Cells were grown in Medium 63 containing 0.2% D-glucose. After being harvested, washed, and suspended in Medium 63, aliquots of 0.50 ml of the resulting suspension (10 mg, dry weight, of cells) were mixed with 1.5-ml portions of Medium 63 or Medium 63 containing sodium polyglutamate, sucrose, or ethanol. After 15 min, \[^{1}H\text{water and inulin}^{[14]C}\text{carboxyl or}^{[14]C}\text{sucrose were added. These labeled suspensions (plus unlabeled controls) were centrifuged to determine the distributions of these compounds as described under "Measurement of Solute Distributions." The data so obtained were used in accord with the scheme in Fig. 1 to compute cell volumes. After centrifugation, the osmotic strength of each supernatant solution was determined with a Knauer freezing point depression osmometer. Total cell volumes are expressed relative to the total volume of cells in Medium 63.

| Composition of extracellular fluid | Osmotic strength of extracellular fluid (mosmol) | \(V_{\text{cell}}\) | \(\frac{V_{\text{cell}}}{V_{\text{total}}}\) |
|-----------------------------------|-----------------------------------------------|----------------|-----------------|
| Medium 63                         | 145                                           | 1.00           | 0.27            |
| Medium 63 + 10% sodium polyglutamate | 428                                           | 0.63           | 0.33            |
| Medium 63 + 1 M sucrose           | 1560                                          | 0.77           | 2.33            |
| Medium 63 + 10% ethanol           | >2000                                         | 1.03           | 0.39            |

proportionality was observed. The reciprocal of the slope of the line, or \(X_{\text{cyt}}/V_{\text{cyt}}\), was 0.36 mosmol/g, dry weight, of cells. The cells used in these experiments were grown in Medium 63; in this case \(V_{\text{cell}}\) was 1.0 and \(V_{\text{cyt}}\) was 1.2 ml/g, dry weight, of cells. The intracellular osmotic strength was, therefore, 300 mosmol \((X_{\text{cyt}}/V_{\text{cyt}})\) in agreement with the values obtained by the other methods.

Fig. 7 shows the results of a similar experiment with the cells suspended in water in place of Medium 63. Under these conditions, the intracellular osmotic strength was estimated to be 165 mosmol, a result not significantly different from the value 179 mosmol obtained with the same cells by the vapor phase equilibration method (Fig. 8), a completely different procedure.

We emphasize that the difference in osmotic strength between cells suspended in water and in Medium 63 represented a volume change, not a significant change in \(X_{\text{cyt}}\). The \(V_{\text{cell}}\) in water was about twice that in Medium 63 (the dry weight yields of cells harvested in water were not significantly different from those harvested in Medium 63). Apparently the cells increased in volume until the osmotically active material was diluted enough to be balanced by \(P_{\text{cell}}\) (see Equations 5 and 8). During this process, \(P_{\text{cell}}\) increased only slightly, from about 3.5 atm in Medium 63 to slightly more than 4 atm in water. The large change in cell volume and small change in \(P_{\text{cell}}\) imply that the cell wall is not a rigid structure in Medium 63, but is extensible.

**Origin of Periplasmic Osmotic Strength**

Under most conditions, *S. typhimurium* cells appeared to have a periplasmic osmotic strength greater than that of their surroundings. The situation with cells in water was the best defined; the osmotic strength of the medium was essentially zero; that of the periplasm approximately 170 mosmol. The sum of the molar concentrations of the material dissolved in periplasmic water must, therefore, have been at least 170 mmolal. Solutes of high molecular weight could not, by any reasonable estimate, be at this concentration within the periplasm. More-
across the outer membrane. Based on Na\(^+\) distribution measurements, \(W_{\text{inner}}\) was then calculated and ranged from 300 to 400 m\(\text{M}\) according to this potential, the ionic composition of the periplasm of cells in water contains 50 mM potassium phosphate.

While the cytoplasmic and outer membranes are fused at several points (14), we presume that the periplasmic space is a continuum bounded on one side by the cytoplasmic membrane and on the other by an organelle containing at least the cell wall and the outer membrane. This organelle will hereafter be designated the exoskeleton to distinguish it from the commonly used term "cell envelope," which also includes the periplasm.

**Table VI**

| Experiment | \(j\) | \(V_{\text{mem}}\) \(V_{\text{inner}}\) | \(E_{\text{mem}}\) | \(\Sigma / j\) | Periplasmic osmotic strength |
|------------|------|------------------|-------------|-------------|--------------------------|
| 1          | Na\(^+\) | 1.97 | 17 | 282 | 209 m\(\text{M}\) |
|            | Cl\(^-\) | 0.35 | 27 | 386 | 285 m\(\text{M}\) |
| 2          | Na\(^+\) | 3.38 | 31 | 419 | 309 m\(\text{M}\) |
|            | Cl\(^-\) | 0.42 | 23 | 304 | 231 m\(\text{M}\) |
| 3          | Na\(^+\) | 3.11 | 29 | 387 | 286 m\(\text{M}\) |
|            | Cl\(^-\) | 0.52 | 30 | 389 | 287 m\(\text{M}\) |

The osmotic strength of comparable concentrations of \(K_2HPO_4\) solutions ranges from 200 to 300 m\(\text{M}\) (11), values comparable with values for the osmotic strength of the periplasm as derived from the methods described above.

Thus, we conclude that the osmotic properties of the periplasm are a consequence of a Donnan equilibrium, and the procedure used here can give a reasonable approximation of the ion composition of the periplasm when cells are suspended in complex media.

**Discussion**

We have attempted in this report to characterize some fundamental properties of the periplasm in the enteric bacteria *Salmonella typhimurium* and *Escherichia coli*. The periplasm is located in a cell compartment, the periplasmic space, which contains a substantial fraction of the cell water, and which is distinct both in polymer and ionic composition from either the cytoplasm or the extracellular medium.

While the cytoplasmic and outer membranes are fused at several points (14), we presume that the periplasmic space is a continuum bounded on one side by the cytoplasmic membrane and on the other by an organelle containing at least the cell wall and the outer membrane. This organelle will hereafter be designated the exoskeleton to distinguish it from the commonly used term "cell envelope," which also includes the periplasm.

**Figure 8.** Intracellular osmotic strength as determined by the vapor phase equilibrium method. Aliquots of 1 ml were removed from the cell suspension used in the experiment described in Fig. 7. These were placed on tared glass cover slips where they were frozen and lyophilized. Each cover slip was then weighed and placed over the center well of a Conway microdiffusion dish containing a solution of sucrose in its outer well. After 1 day (similar results were obtained after 2 days) the cover slips were weighed again. \(W\) corresponds to the weight of water (in kg per g, dry weight, of cells) which had condensed on a cover slip. Details of the vapor phase equilibrium method are given under "Experimental Procedures." The intracellular osmotic strength of cells in water was estimated to be 179 m\(\text{M}\) from an \(X\) (equivalent to \(X_{\text{Na}} + X_{\text{Cl}}\)) of 0.538 mosmol/g, dry weight, of cells and a \(V_{\text{cell}}\) of 3.0 ml/g, dry weight, of cells.

Over, the material could not be of low molecular weight: small molecules would have been lost as the cells were suspended in water. However, if the large solutes were polyvalent ions, then small ions of opposite charge would be concentrated within the periplasm to preserve electroneutrality, and periplasmic concentrations could be maintained high enough to explain the observed osmotic strength. In other words, periplasmic osmotic pressure could originate from a Donnan equilibrium across the outer membrane.

A Donnan equilibrium results in a potential across the membrane because of unequal distribution of each diffusible ionic species, and this potential may be calculated from the Nernst equation (13). At 25° where \(E\) is the membrane potential in millivolts and \(G_{\text{Na}}\) and \(G_{\text{Cl}}\) are the concentrations of an ion of charge \(z\) on opposite sides of the membrane:

\[
E = (59.2z) \log \left( \frac{[j_1]}{[j_2]} \right) \tag{12}
\]

As shown in Table VI, experiments with radiolabeled Na\(^+\) and Cl\(^-\) showed that each of these ions distributed unequally across the outer membrane. From the measured volumes, the potential across the outer membrane (\(E_{\text{mem}}\)) was estimated, and essentially the same values were obtained by using either the Na\(^+\) or Cl\(^-\) distributions. The value for \(E_{\text{mem}}\) was between 17 and 31 mV for cells in Medium 63. Membrane potentials generated by Donnan equilibria are sensitive to the ionic strength of the medium. This was true for the potential across the outer membrane. Based on Na\(^+\) distribution measurements, \(E_{\text{mem}}\) was only about 5 mV for cells in 200 mM sucrose (molol). However, if the large solutes were polyvalent ions, then molecules would have been lost as the cells were suspended in complex media.
the periplasm. The mechanical strength, flexibility, and permeability properties of the exoskeleton largely determine both the composition of the periplasm and the volume it occupies.

When the enteric bacteria are grown under a variety of conditions and are suspended in their growth medium or its equivalent, such as Medium 63, the periplasmic space contains from 20 to 40% of the cell water. Although total cell volumes may change as a function of growth conditions, or stage of growth, or other variables, the ratio of periplasmic to total cell water remained relatively constant. This ratio did not change appreciably even when cells were exposed to metabolic inhibitors such as azide, cyanide, arsenate, and dinitropheno1e, substances which have profound effects on the volumes of mitochondria (15). The limiting volumes of the periplasmic space are defined by suspending the cells in Medium 63 where the periplasm contains as little as 90% of the total cell water, and in 1.0 M sucrose, where the periplasm occupies up to 80% of the total volume.

The exoskeleton is not a rigid structure. Thus, it collapses when subjected to increased osmotic pressure from the outside (medium polyglutamate) and expands slightly when the pressure is increased on the inside (by transferring cells from Medium 63 to water). A simple analogy is to compare the exoskeleton to a dialysis bag. In Medium 63, the exoskeleton is not in its fully expanded state. Under these conditions, it contributes significantly to total pressure applied to the periplasm; that is, the periplasmic osmotic pressure is equal to the sum of the external osmotic pressure and the pressure exerted by the exoskeleton. In hypertonic medium, however, where the exoskeleton is partially collapsed, its contribution to the total pressure on the periplasm may decline to zero.

In relating our measured values for cell volumes (total, periplasmic, and cytoplasmic) to those actually present in the cell, we again emphasize that our data reflect water distributions and do not include either dissolved or insoluble solutes (and organelles). The contributions of the latter to actual volumes are not known. The water distributions appear to be good approximations since essentially the same results were obtained by using several methods and solutes and a minimal number of assumptions (such as the permeability properties of the exoskeleton to inulin, sucrose, etc.). In using this information to measure quantitatively the osmotic pressures of the various compartments, however, a number of simplifying assumptions have been made. The proper thermodynamic method would be to determine the chemical potentials or activities of the solvent (water) in each of the cellular compartments and in the external medium. Since this is not technically feasible, we have resorted to measurements of cell and compartment volumes when cells are placed in solutions of known osmotic pressure. These values were then used along with the van’t Hoff approximation to calculate the osmolarities of the dissolved solutes within the cell compartments, i.e., the concentrations of the ideal solutes within the compartments which would give the same osmotic pressures. In addition, we have assumed in experiments where the external osmotic pressures are altered (as for example with sucrose) that the internal osmolyte compositions remain essentially constant. Thus, our results are approximations of actual pressures and dissolved solute concentrations. However, the agreement between results obtained with quite different methods indicates that our major conclusions are valid.

The permeability properties of the exoskeleton have been extensively studied (16-21). Unlike the cytoplasmic membrane, which is highly selective, the exoskeleton is relatively nonspecific with respect to its permeability except for the size of the solute. In other words, the exoskeleton acts as a sieve, permitting the passage of low but not high molecular weight solutes. It should be noted, however, that anionic polypeptides behaved anomalously (22), as if there were some restriction in their movement across the cell envelope. These findings may possibly be explained by the present results (discussed below).

A major finding presented here is that a Donnan equilibrium exists across the outer membrane, and that this equilibrium results from the presence of a high level of anion within the periplasm which cannot penetrate the exoskeleton. Our findings suggest that this ‘fixed’ anion is present at approximately 150 to 200 μeq/g, dry weight, of cells. For reasons described below, this results in a total osmotic strength of solute within the periplasm of approximately 300 mosm when cells are suspended in Medium 63. (It is of interest to note that the comparable value for human blood plasma is approximately 290 mosm (23.) The nature of the fixed anion in the periplasm is not known, but some information is available on the composition of the periplasm. Early work (4, 24) showed that intact bacterial cells could cleave extracellular sugar phosphate esters. It was subsequently demonstrated that the periplasm contains a number of hydrolases (3) and solute binding proteins (25). The latter in particular are present at high levels in induced cells. Conceivably, the sum total of these proteins contributes substantially to the total fixed anionic charge in the periplasm.

The exoskeleton and inner membranes of E. coli and S. typhimurium possess significant negative charge. These are found in the dicarboxylic acids of the peptidoglycan component (26), in the phosphate and 2-keto-3-deoxyoctonate components of the lipopolysaccharide (27), and in the anionic lipids of the inner membrane (28). It is not clear that all of these potential charged groups are free to contribute to the anionic charge within the periplasm. Some of these groups, perhaps a large fraction, may form a tight complex with divalent cations such as Mg2+ and thus would not be available to contribute to the Donnan potential. A rough estimate shows, furthermore, that even if these groups were freely available to the cations within the periplasmic space, they would still not account for all of the fixed anionic charge. Of the 150 to 200 μeq of net negative charge per g dry weight of cells calculated to be present in the periplasm, approximately 10% would be contributed by lipopolysaccharide (28, 29), 10% from the cell wall (30), and 5% from the phospholipids of the inner membrane (28), or a total of about 25% of the measured value. It seems reasonable to suggest that other polyanions, perhaps even nucleic acids, provide a major source of this net negative charge.

As a result of the fixed anions, a Donnan equilibrium exists across the outer membrane, and this equilibrium is largely responsible for the osmotic pressure in this space (which is equal to the osmotic pressure of the cytoplasm). The Donnan potential across the outer membrane is negative inside and varies with the concentration of salt in the medium. In Medium 63, the potential was 20 to 30 mV, calculated on the basis of Na+ and Cl− distributions. In phosphate buffer, ranging from 5 to 200 mM, the potential changed from 80 to 5 mV. The Donnan potential results in an asymmetric distribution of all ions between the periplasm and the extracellular medium. For example, in Medium 63, the ratio of periplasmic...
to external Na\(^+\) was about 3, while for Cl\(^-\) it was about 0.3. Based on these measured distributions and the calculated potential, and using the Nernst equation, the distributions of all other ions in Medium 63 were estimated. The total calculated ionic strength of the periplasm accounted for its experimentally determined osmotic pressure.

The physiological consequences of the Donnan equilibrium are not known, but several speculations can be offered.

1. The ionic composition of the periplasm can differ substantially from the external medium, particularly in media of low ionic strength. The periplasm, therefore, acts as a "homeostatic" compartment to protect the cytoplasm. We emphasize that the asymmetric distributions increase with charge of the ion to comply with the Donnan equation. The calculated Mg\(^{2+}\) distribution in the experiment cited above was 9, for example, when the Na\(^+\) distribution was 3. For a polyanion, such as polyglutamate, the anion would be largely excluded from the periplasmic space under equilibrium conditions (22) even where it is small enough to penetrate the exoskeleton.

2. Mitchell (31) has suggested that protons play a pivotal role in many bacterial solute transport systems. The driving force in these systems is the "protonmotive force," which is composed of two elements, the cytoplasmic membrane potential and the pH gradient across the membrane. Work with whole cells and with vesicles supports the Mitchell theory (for a review, see Ref. 32). The Donnan equilibrium across the outer membrane should be considered in such experiments, particularly those involving whole cells. First, the fixed anions in the periplasmic space may act as a substantial buffer in this compartment. Second, protons will distribute just as any other cations across the outer membrane in accord with the Nernst equation. Thus, the pH in the periplasm should be significantly below that in the medium.

3. The potential across the outer membrane could significantly affect the functions of surface organelles such as flagella. If true, the rapid (even small) changes in the potential could be physiologically significant. For example, the binding of a solute to its periplasmic binding protein may alter the charge on the protein and in this way alter the fixed anionic charge and thus the membrane potential.

In summary, the periplasm comprises a substantial cell compartment. In view of its fixed anions and the accompanying potential across the outer membrane, the periplasm may play a much more significant role in bacterial cell physiology than currently recognized.

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Periplasmic space in Salmonella typhimurium and Escherichia coli.
J B Stock, B Rauch and S Roseman

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Periplasmic space in *Salmonella typhimurium* and *Escherichia coli*.

*Jeffry B. Stock, Barbara Rauch, and Saul Roseman*

Page 7851 (Growth and Collection of Cells)

The basic salts medium used for growth of cells is that routinely used in this laboratory (Saier M. H., Jr., Simoni, R. D., and Roseman, S. (1976) *J. Biol. Chem.* 251, 6584–6597). It is a modification of Medium 63 and contains: 50 mM KH₂PO₄, 15.1 mM (NH₄)₂SO₄, and 0.81 mM MgSO₄; the pH is adjusted with KOH to 7.3.

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d(m⁴ATP) as a probe of the fidelity of base incorporation into polynucleotides by *Escherichia coli* DNA polymerase I.

*James Douglas Engel and Peter H. von Hippel*

In the “Discussion” we indicated that synthesis of DNA by DNA polymerase I is primarily dispersive. Studies have recently come to our attention (Uyemura, D., Bambara, R., and Lehman, I. R. (1975) *J. Biol. Chem.* 250, 8577–8584; Bambara, R. A., Uyemura, D., and Lehman, I. R. (1976) *J. Biol. Chem.* 251, 4090–4094; and especially Bambara, R. A., Uyemura, D., and Choi, T. (1978) *J. Biol. Chem.* 253, 413–423) which show quite convincingly that, on a microscopic level, synthesis by DNA polymerase I is actually processive under almost all conditions.

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