Permeability Properties of *Rickettsia mooseri*

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The passive permeability properties of *Rickettsia mooseri* to both inorganic and organic solutes have been examined. Visual observations by phase-contrast microscopy of rickettsiae in macerated yolk sacs taken directly from heavily infected eggs revealed plasmolysis with hypertonic NaCl and KCl as well as with sucrose solutions. In contrast, similar visual studies of rickettsiae which had been subjected to freezing or to a purification process, or both, were plasmolyzed by hypertonic sucrose but not by hypertonic NaCl and KCl. These primary observations were extended to a variety of solutes and were placed on a quantitative basis by use of optical density and radioisotope dilution methods. Intracellular Na\(^+\) and K\(^+\) concentrations in processed rickettsiae, measured by flame photometry, closely paralleled the concentration of these ions in the suspending medium. It was concluded that *R. mooseri* appears to possess an osmotically active, functional, and structural membrane distinct from the cell wall, located at the surface of a structure analogous to the bacterial protoplast. In the intact organism, this membrane is passively impermeable to sucrose, NaCl, and KCl. However, altered permeability properties, especially to inorganic electrolytes, may be expected in rickettsiae which have been stored in the frozen state and subjected to a lengthy purification process.

The basis for the apparent obligate nature of intracellular parasitism among pathogenic rickettsiae remains unknown. With their relatively complex structural, chemical, and metabolic properties, they resemble small, fastidious bacteria in many ways. Thus, they appear to maintain their morphological integrity during growth and multiplication (27), they appear to oxidize certain substrates through recognized pathways to yield high energy bonds (5, 34), and they appear to incorporate some amino acids into proteins from special cell-free media (8, 9). They very likely contain the enzymatic equipment necessary to synthesize major cell components from simpler substances.

Rickettsiae may depend upon host cells for some unique essential nutrient(s). Simple provision of nutrients, however, is only one category of the prerequisites for cell growth. Qualitatively different kinds of phenomena might be involved, for example, the maintenance of an internal milieu favorable to metabolic processes. In free-living cells, this essential function is performed by or at the cell membrane by various processes which include selective passive permeability, stereospecific active transport systems, "pumping" mechanisms, etc. (26).

The present study was undertaken to explore the idea that one point of dependence upon a host cell of a rickettsia might lie in the realm of cell membrane functions; that is, rickettsiae might be deficient in one or more of the cell membrane regulatory functions required for existence as "free-living" organisms. The host-cell membrane could conceivably perform the necessary regulatory functions for parasitic organisms residing within it.

This study leans strongly on the phenomenon of plasmolysis (i) to demonstrate the existence of a functional cell membrane within the cell wall of *Rickettsia mooseri* (*Rickettsia typhi*), and (ii) to reveal some of the permeability properties of this membrane. The results indicate that the permeability properties of the rickettsial cell membrane are similar in many respects to the membrane properties of a "free-living" cell like *Escherichia coli*.

**Materials and Methods**

Suspensions of *R. mooseri* were prepared as described by Wisseman et al. (33) from frozen (−60 to −70°C) infected yolk sacs, except that SPG [sucrose-phosphate-glutamate solution of Bovarnick et al. (7)]
was the suspending medium throughout the process. Rickettsiae, recovered from about 70 g of starting yolk sac material, were generally suspended in 5 to 10 ml of SPG at the end of the process. They were employed immediately after final resuspension. Table 1 records some characteristics of the suspensions employed in this study.

Logarithmic-phase Escherichia coli strain B was obtained from Trypticase Soy Broth (BBL) cultures grown for 4 hr (unless stated otherwise) at 37 C on a reciprocal shaker. The standard inoculum was a heavy suspension of stationary-phase cells which had previously been frozen in small samples and stored at -70 C. The sedimenterodlogistic-phase cells were resuspended in the desired volume of supernatant culture medium without washing.

Radioisotope procedure. A radioisotope dilution method was devised for measuring plasmolysis of rickettsial and bacterial cells. It depends upon differences in penetration through the cell wall by inulin and sucrose. The technical procedure is described here, and the theory and verification are presented below.

Suspensions of either organism (about 40 mg (wet weight) of cells per ml) were dispensed in 0.5-ml quantities to 12 Spinco cellulose tubes (5 by 1.5 inches (ca. 0.5 by 1.6 cm)). Centrifugation in a Spinco SW39L swinging-bucket rotor (4 tubes per bucket) at 44,000 x g for 20 min at ambient temperature was followed by discarding the supernatant fluids and swabbing the tubes (walls and pellets) with rolls of Whatman no. 5 paper to minimize carry-over fluid. The packed cells, suspended in 6.6 ml of various osmotic solutions, were added to tubes containing 0.1 μC (0.2 μc/μmole) of dried, uniformly labeled sucrose-14C or carboxyl-labeled inulin-13C (Calbiochem, Los Angeles, Calif.). Solute in various osmolalities were made up in 0.01 M phosphate buffer at pH 7.4, but, in the case of R. mooseri, these test solutions were also contaminated with 10-4 M adenosine triphosphate (ATP), 3 × 10-4 M nicotinamide adenine dinucleotide (NAD), and 2 × 10-4 M MgCl2. The cell suspensions were shaken for 10 min at 35 C, a period of time sufficient, as determined in preliminary experiments, to allow equilibration of the radioisotopic solutes throughout their permeable volumes. The suspensions were then transferred to small preweighed cellulose presurture tubes (as above) and centrifuged at 44,000 x g for 30 min at ambient temperature. The supernatant fluids were promptly removed from the tubes, and the pellets and tube walls were swabbed with a series of dry, wet, and dry rolls of paper. The tubes, with their pellets, were weighed to 0.01 mg and packed cell weight was determined by difference. A 0.1-ml amount of cell suspension was plated in triplicate on minimal agar (M9, 0.35% glacial acetic acid) a fluid was plated on 3-cm planchets for counting in a Nuclear-Chicago gas-flow counter with a Micromil window; the undiluted portion of the supernatant fluid was analyzed with a Model G Fiske Osmometer for osmolality. The packed cells were diluted to 2 ml with water. A 0.1-ml amount of cell suspension was then placed on a wetting agent plate (Filter-aid) for counting at infinite thinness. A total of 10,000 counts was recorded for each plated sample and the counts per minute were corrected for background.

Spectrophotometric procedure. This method was based on the well-known phenomenon of increase in optical density when suspensions of cells, protoplasts, or mitochondria are placed in solutions of increased osmolality. The basis for considering it to be a measure of plasmolysis is presented elsewhere in this paper. The procedure only is described here. To detect plasmolysis by optical-density measurements, 0.1 ml of the freshly prepared cell suspensions (as above) was added to 3 ml of test solution. The cell suspensions were previously adjusted so that this dilution in low osmolality solution resulted in an optical density at 650 μμ of about 0.2 with a Beckman DU spectrophotometer. Readings were made at room temperature 1 min after addition of the cells. The test solutes were dissolved in 0.01 M phosphate buffer at pH 7.4 and the pH was readjusted to 7.4 when necessary. The test solutions were made on an osmolality basis, taking into consideration the osmotic components of the various solutes, to obtain solutions of equal osmotic properties. Corrections for the diminution of optical-density values of suspensions by the high refractive indices of test solutions were made by use of Ficoll (Pharmacia, Inc., New Market, N.J.), a high molecular weight sucrose polymer. Refractive indices of water, 1.335; of Ficoll, 1.456. A 2.5 cm Bausch & Lomb refractometer. The application of the method is shown in the Results section.

Intracellular sodium and potassium level as determined by flame photometry. Both E. coli and R. mooseri suspensions were prepared as described above. For use in these experiments, the cells were first centrifuged by centrifugation for 15 min at 14,000 X g in a Spinco no. 40 rotor, followed by resuspension in an appropriate small volume of the same medium (Tryp-
To obtain equivalent masses of cells for exposure to different solutions in any given experiment, 0.5-ml portions of these concentrated suspensions were distributed to 3/4 g by 15/4 inch tubes and the cells were sedimented by centrifugation at 44,000 × g for 15 min in a Spinco SW39L rotor. The packed cells were drained of supernatant fluid, and all possible traces of suspending medium were removed by the filter-paper-swabbing technique described above. The packed cells were then suspended in the desired solution and incubated with shaking for varying lengths of time in a Warburg apparatus. Duplicate flasks were then pooled (6 ml), and the cells were concentrated to 0.5 ml in a Spinco no. 40 rotor (36,000 × g, 15 min). The cells were then sedimented (44,000 × g, 30 min) in an SW39L rotor. The packed cells were drained as previously described, and the wet weights were determined. All operations were performed at room temperature. The packed cells and supernatant fluids were appropriately diluted to determine their K+ and Na+ concentrations by flame photometry (Baird Associates, Inc., Cambridge, Mass.; flame photometer, internal lithium standard). Two determinations were made to correct the Na+ and K+ values obtained in the E. coli pellets for the volume of extracellular fluid trapped in the pellet and to convert the values from milliequivalents per gram to milliequivalents per milliliter. One was a determination of the free space in an E. coli pellet centrifuged under the conditions described above. From the isotopic data with E. coli, it was shown that plasmolysis experiments described in this report, an average value of 50% of the pellet as sucrose-permeable volume was obtained. This value was used to correct all the Na+ and K+ data obtained on E. coli.

The specific gravity was obtained by suspending a weighed pellet in a known volume or weight of water. By determining the weight of a unit volume of this suspension, the volume of the total suspension was determined. Subtraction of the volume of added water gave the pellet volume, and its specific gravity, which was 1.05, was thus determined.

The specific gravity determination allowed conversion from milliequivalents of K+ or Na+ per gram of packed cells to milliequivalents of K+ or Na+ per milliliter of packed cells.

Since the sucrose-permeable volume of the pellet, i.e., all that volume that is outside of the cytoplasmic membranes of the cells, was known, and the electrolyte concentration of the supernatant fluid was known, the K+ and Na+ concentrations of the pellet could be corrected to give the true cytoplasmic concentration of these components.

In the case of the rickettsial packed cells, no specific gravity measurements were made, and no corrections for this factor or for sucrose-permeable space have been applied to the data presented for the rickettsiae. This was not believed necessary, since the results showed comparable intracellular and extracellular levels of Na+ and K+, a situation where the correction for extracellular fluid would be relatively insignificant.

**RESULTS**

Direct visual demonstration of plasmolysis in *Rickettsia mosseri and Escherichia coli*. By direct visual examination of the wet films under phase-contrast microscopy, plasmolysis was readily demonstrated with the bacterium, *E. coli*. Plasmolysis occurred when this organism was suspended in a variety of solutions of inorganic salts and organic compounds, including NaCl, KCl, and sucrose, ranging in tonicity from 0.2 to 0.6 osmolar. Solutions of glycerol, dimethyl sulfoxide, or urea did not produce plasmolysis with this organism. These results are in general agreement with the observations of others on this and other bacteria (20), and serve to demonstrate the adequacy of the method.

When fresh yolk sacs heavily infected with *R. mosseri* were taken directly from incubating eggs, ground to a fine homogenate in a mortar without freezing or storage, diluted immediately in the desired solution, and examined under the phase-contrast microscope, plasmolysis was detected with 0.6 osmolar solutions of NaCl, KCl, and sucrose made up in 0.01 M phosphate buffer (pH 7.2). Thus, the behavior of these fresh rickettsiae was similar to that of the E. coli cells.

In contrast, rickettsiae which had been stored in the frozen state at about −70 C, thawed, and processed through a complex purification procedure exhibited very different differences in plasmolysis, and hence permeability, from the fresh organisms. Thus, although plasmolysis was regularly produced by 0.6 osmolar sucrose, no plasmolysis was detected in the presence of 0.6 osmolar NaCl or KCl. The storing and processing of rickettsiae seemed to have caused a differential loss of the cell membrane to regulate the penetration of different solutes into the cell.

These direct visual observations, though only of a qualitative nature, permit the following inferences to be drawn regarding *R. mosseri*. (i) The shrinkage of a protoplast-like structure within the rigid cell wall in the presence of hypertonic solutions strongly suggests that this organism possesses a functional, and possibly a structural, selectively permeable cell membrane within, and distinct from, the cell wall. (ii) Like the bacterium *E. coli*, fresh *R. mosseri*, immediately after liberation from infected host cells, are plasmolyzed by, and hence are impermeable to, NaCl, KCl, and sucrose. (iii) The process of storing and purifying rickettsiae, as is commonly done for metabolic studies, causes differential changes in membrane regulatory function, resulting in the loss of capacity to exclude the electrolytes, NaCl, and KCl, but not the larger organic molecule, sucrose.
Quantitative studies of plasmolysis and cell membrane permeability in purified R. mooseri and E. coli. Previously, most metabolic studies of rickettsiae have been made with suspensions which have undergone freezing, thawing and purification. The altered permeability of such processed organisms was studied here by quantitative techniques to define more clearly the potential influence which this artificial cell membrane property might have on the results obtained in such studies and, in turn, on the inferences which might be made about the nature of these organisms. Several different techniques were employed for this purpose.

Detection of plasmolysis by radioisotope dilution method. Three distinct spaces have been recognized in a centrifuged pellet (11): the intercellular space, an outer cellular space, and a central region (see Fig. 1). Different kinds of molecules show differential penetration into the various spaces. Measurement of plasmolysis by the radioisotope dilution method depends upon the differential permeability of cell wall and cytoplasmic membrane to 14C-labeled inulin and sucrose, and the calculation of the desired volumes from the isotope dilution. Two fundamental premises of the method are as follows.

(i) Inulin does not penetrate the cell wall but is otherwise uniformly distributed in the fluid between cells and in the supernatant fluid over the packed cells (11, 15, 17, 32).

(ii) Sucrose penetrates the cell wall but not the cell membrane. Conway and Downey (11) showed that certain nonmetabolized sugars were distributed in a larger volume than the intercellular space measured with inulin but did not penetrate the central area. Neither R. mooseri nor the strain of E. coli employed metabolize sucrose. Moreover, neither direct visual observation nor optical measurement of either kind of cell plasmolyzed by sucrose indicated deplasmolysis over a period of at least 1 hr. Hence, it is unlikely that sucrose penetrates the cell membrane. There is, of course, the question of what percentage of the cell wall space is actually permeable to sucrose. However, this must be relatively high, considering the good agreement between cell wall space based on isotopic data and that derived from measurements of cell wall thickness as seen in electron micrographs of thin sections.

The possibility of surface adsorption of radioactive inulin or sucrose onto cells could not be categorically excluded, but it is difficult to conceive of how this could be closely correlated with varying osmotic pressures produced by a variety of inorganic and organic solutes.

On the basis of these assumptions, the volume of fluid permeated by sucrose in a pellet would be the sum of the following components: intercellular fluid, cell wall, and space between cell wall and cell membrane (see Fig. 1). Since intercellular volume can be determined by independent means (inulin), it is possible to calculate the relative volume of the cell penetrated by sucrose (sucrose-permeable volume), which is composed of the cell wall and the space between cell wall and cell membrane. In the unplasmolyzed cell, it can be assumed that the space between cell wall and cell membrane is negligible; hence, under these conditions sucrose-permeable volume would approach cell wall volume.

The derived values can be obtained from a set of simple equations in which directly measured experimental values can be substituted. Thus, if \( V_{\text{esp}} \) = volume of sucrose pellet (microliters); \( V_{\text{epi}} \) = volume of inulin pellet (microliters); \( V_s \) = volume of sucrose-permeable space in sucrose pellet (microliters); \( S_{\text{ep}} \) = counts per minute for total pellet with sucrose-14C; \( S_{\text{up}} \) = counts per minute per microliter of supernatant fluid with sucrose-14C; \( I_{\text{ep}} \) = counts per minute for total pellet with inulin-14C; \( I_{\text{up}} \) = counts per minute per microliter of supernatant fluid with inulin-14C; \( S \) = per cent of pellet permeable to sucrose-14C; \( S P V \) = per cent of cell penetrable by sucrose, then

\[
V_s = \frac{S_{\text{ep}}}{S_{\text{up}}} \quad (1)
\]

and

\[
S = \frac{V_s}{V_{\text{esp}}} \times 100 \quad (2)
\]

In a similar manner

\[
V_i = \frac{I_{\text{ep}}}{I_{\text{up}}} \quad (3)
\]

**FIG. 1.** Schematic diagram of centrifuged plasmolyzed rickettsial or bacterial cells. Symbols: \( \bigcirc \) = inulin, intercellular space; \( \bullet \) = sucrose, intercellular space + cell wall space + protoplast shrinkage space.
and

\[ I = \frac{V_1}{V_{w1}} \times 100 \]  

(4)

Since two separate pellets are required for each determination, the assumption has been made that the centrifugal force on each was equivalent, since they were spun simultaneously at the same radius with approximately equal volumes of cell suspension.

With this assumption, then

\[ SPV = \frac{(S - I)}{(100 - I)} \]  

(5)

which is equal to the percentage of the total cell volume made up by cell wall volume and volume between cell wall and cell membrane. With increasing plasmolysis this value should increase.

The results obtained with various solutes with \( R. \) \( mooseri \) and \( E. \) \( coli \) are recorded in Table 2. They show certain deviations from the ideas which are inherent in this method when applied to either \( R. \) \( mooseri \) or \( E. \) \( coli \). Thus, with increasing osmolality of the suspending medium, the packed cell wet weight and the inulin-\(^{14}C \) space decreased, most likely from both loss of turgor pressure in plasmolyzed cells and compression of the cell walls during centrifugation. If this mechanical distortion also impinged upon the space between the protoplast and the cell wall, the measured sucrose-permeable spaces might actually tend to be smaller than in the idealized model with no distortion. A similar phenomenon has been observed by others in plasmolyzed \( E. \) \( coli \) (23) and in butanol-treated \( Staphylococcus \) \( aureus \) (19).

Despite this potential systematic source of error, the method yielded generally expected results with \( E. \) \( coli \) (Fig. 2), i.e., about an equal degree of plasmolysis with hyperosmotic solutions of both salts and sucrose. These results indicate relative impermeability of the bacterial cell membrane to both classes of solutes. In contrast, sucrose, but not KCl or NaCl, plasmolyzed processed \( R. \) \( mooseri \) (Fig. 3, Table 2). This indicated that the rickettsial cell membrane was relatively impermeable to sucrose but was highly permeable to the salts. Regular results showing expected trends were obtained only when the plasmolyzing media were supplemented with MgCl\(_2\), ATP, and NAD (see Materials and Methods), substances previously shown to help maintain metabolic integrity of purified rickettsial suspensions (6).

The cell wall volumes, i.e., the sucrose-\(^{14}C \)-permeable cell volume in unplasmolyzed cells, were 20 and 30% of the total cell in \( R. \) \( mooseri \) and \( E. \) \( coli \), respectively. These values agree with cell wall volumes of bacteria reported previously (26), and with results obtained in other studies in these laboratories with \( R. \) \( mooseri \) cell walls (Wood and Wiseman, to be published). The space created by maximal plasmolysis in both organisms is about 20% of the total cell volume and about 25% of the cytoplasmic volume. The osmolality of sucrose which caused 50% of the total cytoplasmic shrinkage was 0.2 osmolar (4.5 atm) in the rickettsiae and 0.28 osmolar (6.3 atm) in \( E. \) \( coli \). The \( E. \) \( coli \) figure agrees with the observations of others (20) obtained by different means.

Plasmolysis detected by optical-density measurements. Generally, the capacity to cause optical-density increases was determined with the solutes at 0.05 and 0.6 osmolar concentrations which were assumed to yield negligible and maximal plasmolysis, respectively, with nonpermeating solutes.

**Table 2. Derivation of Rickettsia mooseri sucrose-permeable volumes as affected by osmolality* of the suspending medium**

<table>
<thead>
<tr>
<th>Measured osmolality*</th>
<th>Sucrose-(^{14}C )</th>
<th>Inulin-(^{14}C )</th>
<th>Vol of cell penetratrated by sucrose (( S - I ))/(100-( I ))</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Counts/min</td>
<td>S (sucrose space in pellet)</td>
<td>Mag</td>
</tr>
<tr>
<td>0.10</td>
<td>116.3</td>
<td>304.9</td>
<td>16.86</td>
</tr>
<tr>
<td>0.15</td>
<td>118.4</td>
<td>299.3</td>
<td>16.02</td>
</tr>
<tr>
<td>0.25</td>
<td>103.1</td>
<td>297.3</td>
<td>14.17</td>
</tr>
<tr>
<td>0.35</td>
<td>120.7</td>
<td>308.2</td>
<td>15.00</td>
</tr>
<tr>
<td>0.50</td>
<td>113.9</td>
<td>279.4</td>
<td>15.45</td>
</tr>
<tr>
<td>0.60</td>
<td>118.5</td>
<td>293.7</td>
<td>15.35</td>
</tr>
</tbody>
</table>

* In this experiment, sucrose was used to vary the osmotic pressure of the solution.

* Given to the nearest 0.05 osmolar.

* For calculations, the specific gravity of pellet was assumed to be 1.0.
Table 3 summarizes the optical effects of a variety of solutes on both organisms. Glycerol and dimethyl sulfoxide, known to be rapidly penetrating solutes for other organisms, caused only slight optical effects in E. coli and R. mooseri. E. coli was relatively impermeable to the remainder of the solutes, all of which produced approximately the same optical effect, indicating equal amounts of plasmolysis caused by a variety of solutions with equal osmotic properties. In the rickettsiae, the salts gave optical effects about half of that obtained with the sugars sucrose, glucose, and ribose, and the amino acids tested were intermediate in their effects. In addition, single experiments employing NaSO₄, NaBr, KI, KNO₃, and NH₄Cl with R. mooseri and E. coli revealed that the salt permeability of each organism followed the pattern set by the other salts shown in Table 3. The optical effect in both organisms was prevented by pretreatment of the cells with 5% (v/v) butanol or heating at 60 C for 30 min.

The effect of freezing and thawing on cell permeability is known to vary greatly with the nature of the menstruum in which the cells are frozen. When fresh, never frozen, purified rickettsiae (Table 3) were treated with the same series of solutes, the same high salt permeability was noted; consequently, this phenomenon cannot be ascribed to the freezing procedure alone. However, these rickettsiae had been subjected to chilling and a lengthy purification process, factors which affect permeability in bacteria as well (29-31).

The masking of the optical-density increase by high refractive index solutions, generally ignored by other investigators, was eliminated by the use of Ficoll (Fig. 4). The Ficoll correction curve, determined for each cell preparation used, always showed a linear drop-off in optical density value of the cell suspension with increased refractive index due to Ficoll. The levels of this high molecular weight substance (100,000) used were from 0 to 17% (w/w). It should be pointed out that 17% Ficoll is only 2 x 10⁻³ M, and thus has little osmotic effect. Figure 5 shows the manner in which corrections were made for the refractive indices of the test solutions, by use of the Ficoll correction curve.

Compatibility of results obtained by the radioisotopic and optical-density techniques. Gilby and Few (12) showed that protoplasts of Micrococcus lysodeikticus behave as osmometers and follow the van't Hoff-Boyle law at osmotic pressures above the threshold for lysis when such measurements are made by optical-density values of the suspensions. This law can be expressed as

\[ \pi (V - V_0) = \text{constant} \]

where \( \pi \) is the osmotic pressure of the medium, \( V \) is the cytoplasmic volume of the cell, and \( V_0 \) is that cytoplasmic volume of the cell which does not respond to osmotic influences. The ratio \( V_0/V \) will approach unity in a fully plasmolized cell.

From the data in Fig. 2 and 3, \( V_0/V \) has been calculated at osmolarities ranging from 0.1 to 0.6. These data represent actual measurements by radioisotopes of cytoplasmic volume as affected by osmotic pressure. The calculation of \( V_0/V \) involves determination of \( V_0 \), which is 100 minus per cent of cell as sucrose-permeable volume at the 0.6 osmolality (fully plasmolized cells), and \( V \), which is 100 minus per cent of cell as sucrose-permeable volume at a lower measured osmolality. Table 4 shows \( V_0/V \) values for both R. mooseri and E. coli determined in this manner.
TABLE 3. Plasmolysis of Rickettsia mooseri and Escherichia coli as measured by optical effect

<table>
<thead>
<tr>
<th>Solute</th>
<th>R. mooseri</th>
<th>E. coli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Expt 93</td>
<td>Expt 91</td>
</tr>
<tr>
<td>Sucrose</td>
<td>.36</td>
<td>.36</td>
</tr>
<tr>
<td>Glucose</td>
<td>.38</td>
<td>.36</td>
</tr>
<tr>
<td>Ribose</td>
<td></td>
<td>.32</td>
</tr>
<tr>
<td>Glycine</td>
<td>.25</td>
<td>.25</td>
</tr>
<tr>
<td>L-Serine</td>
<td>.32</td>
<td>.29</td>
</tr>
<tr>
<td>L-Valine</td>
<td>.38</td>
<td>.34</td>
</tr>
<tr>
<td>L-Glutamate</td>
<td>.24</td>
<td>.29</td>
</tr>
<tr>
<td>NaCl</td>
<td>.20</td>
<td>.22</td>
</tr>
<tr>
<td>KCl</td>
<td>.17</td>
<td>.20</td>
</tr>
<tr>
<td>KHCO₃</td>
<td>.18</td>
<td>.24</td>
</tr>
<tr>
<td>KH₂PO₄, KH₂PO₄</td>
<td>.18</td>
<td>.21</td>
</tr>
<tr>
<td>Glycerol</td>
<td>.09</td>
<td>.09</td>
</tr>
<tr>
<td>Dimethyl sulfoxide</td>
<td>.10</td>
<td>.06</td>
</tr>
</tbody>
</table>

* Experiment 93 was performed with freshly harvested, never frozen rickettsiae. The other rickettsial preparations had been previously stored at -70 C.

E* = [optical density (650 mp) at 0.6 osmolar minus optical density (650 mp) at 0.05 osmolar] / optical density (650 mp) at 0.05 osmolar. Optical density at 0.6 osmolar corrected for refractive index shift.

By use of the equations of Gilby and Few (12), the optical-density data obtained in typical optical-effects experiments were converted into Vₒ/V values (Table 4). A plot of osmolality versus osmolality/optical density gave a straight line, the slope of which multiplied by optical density gave Vₒ/V at the osmolality of the optical-density value.

The Vₒ/V values obtained from the direct measurement of cytoplasmic volume by radioisotopes and by the equations of Gilby and Few (12) agree quite well, thus providing some confirmation of the validity of the latter's assumption, i.e., that the optical density of a protoplast suspension is inversely proportional to the average protoplast volume. Also, the osmotically inactive cytoplasmic volumes of unplasmolysed R. mooseri and E. coli obtained by both techniques are close to the Vₒ/V reported by Gilby and Few (12) for M. lysodeikticus protoplasts.

Intracellular sodium-potassium regulation in stored and purified rickettsiae. Confirmation of the hypothesis derived from the plasmolysis studies—namely, that stored and purified R. mooseri cells are permeable to Na⁺ and K⁺—was obtained by direct measurement by flame photometry of the intracellular concentration of these ions and correlation of the changes in intracellular concentration with alteration in electrolyte content of the suspending medium. A series of experiments were carried out with purified R. mooseri suspensions (see Materials and Methods for procedural detail).
in which osmotic pressure, Na\(^+\), and K\(^+\) concentration of the suspending medium and metabolic activity of the rickettsiae were manipulated independently. Results of these experiments, recorded in detail in Table 5, have been interpreted as follows.

(i) Changes in osmotic pressure per se in the suspending medium of sufficient magnitude to cause plasmolysis, caused by varying the sucrose concentration, did not influence significantly intrarickettsial concentration of Na\(^+\) or K\(^+\).

(ii) The addition of an oxidizable substrate (glutamate), accompanied by demonstrable oxygen uptake, did not significantly alter the intrarickettsial concentration of Na\(^+\) or K\(^+\).

(iii) Intrarickettsial concentration and ratio of Na\(^+\) and K\(^+\) varied with, and closely approximated, that of the suspending medium.

Parallel experiments with E. coli, a bacterium capable of growing under a wide range of environmental conditions, revealed the expected (26) regulation of internal Na\(^+\) and K\(^+\) concentration, especially in the presence of the oxidizable substrate, glucose.

**DISCUSSION**

Direct visual examination of rickettsiae from freshly harvested, unprocessed yolk sacs disclosed that plasmolysis occurred in hypertonic solutions of various electrolytes and of certain organic substances, but not others, in a manner similar to that observed with the bacterium E. coli. These observations suggested that this intracellular parasite possesses a functional and, probably, an anatomical cell membrane within the cell wall, much like the cell membrane of bacteria in regard to passive permeability properties. They do not support, but do not necessarily exclude, the original hypothesis that the apparent obligate nature of the intracellular parasitism of R. mooseri resides in a defect in parasite-cell membrane function which is supplied by the host-cell membrane.

Most of the metabolic studies performed with rickettsiae in the past have been made with suspensions of organisms which have been subjected,

**TABLE 4. Comparison of per cent of cell cytoplasmic volumes as osmotically inactive volumes (\(V_a/V\)) as determined by the isotopic and optical techniques**

<table>
<thead>
<tr>
<th>Osmolality</th>
<th>Rickettsia mooseri</th>
<th>Escherichia coli</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Optical</td>
<td>Isotopic ((V_a/V))</td>
</tr>
<tr>
<td>0.05</td>
<td>0.76</td>
<td>0.72</td>
</tr>
<tr>
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<tr>
<td>0.35</td>
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\(V_a/V\) = osmolality of inactive cell cytoplasmic volume/actual cell cytoplasmic volume at given osmolality.

Data are from typical optical-effect experiments with sucrose.

Data are derived from Table 3, average of sucrose values.

Data are derived from Fig. 3, sucrose values.

Data are derived from Fig. 2, sucrose values.
of practical necessity, to a variety of cycles of freezing and thawing and purification procedures. Rickettsial cells so processed seemed to have unusual permeability properties with regard to added extracellular metabolites, to leak large molecular substances, and to be sensitive to extracellular electrolytes (5-7, 10, 14, 34). Hence, it was pertinent to examine directly the permeability properties of rickettsiae which had been subjected to these procedures.

Direct visual observation under phase-contrast microscopy revealed that, indeed, purified rickettsiae seemed to have undergone selective alterations in permeability when compared with the fresh rickettsiae. Thus, although passive impermeability (as shown by plasmolysis) was retained for certain organic molecules, plasmolysis now failed to occur with hypertonic electrolyte solutions. This strongly suggested that the purified cells had been injured in some way so as to have lost the capacity to regulate their internal electrolyte composition, one of the earliest manifestations of certain kinds of cell damage (22).

An additional factor affecting the ability of a cell to regulate its electrolyte composition relates to the growth phase. Schultz and Solomon (28) showed that, in contrast to cells in the logarithmic phase, stationary-phase cultures of E. coli tended to lose this ability. This does not negate, however, the importance of freeze-thaw damage as a causative factor in altered cell permeability.

This differential alteration in permeability could account for some of the observations made in various earlier studies on the influence of the electrolyte composition of the suspending medium on metabolic processes in purified rickettsial suspensions (7-9). Hence, certain permeability properties of such cells were described in quantitative terms to define more clearly the kind of cell preparation which has been the subject of considerable investigation and from which most of our knowledge of rickettsial metabolism stems.

Isotopic analysis of the packed cells in the determination of cellular permeable spaces is a more direct approach, and is inherently more sensitive, than analysis dependent upon measurement of solute concentration of the supernatant fluid. The optical-density method, although a less direct approach to cytoplasmic volume changes, has the advantages of allowing observations over any desired time interval and of being highly conservative of rickettsial material. This latter method has been applied by other workers to whole cells (2-4, 13, 18, 20, 21, 24, 25) and to protoplasts or spheroplasts (1, 12, 20). The data presented here support the idea that the optical density of a plasmolyzed cell suspension bears an inverse relationship to the cytoplasmic volume, and that the cytoplasmic component of the cell behaves as an osmometer at osmotic pressures above the threshold for plasmolysis. The values obtained from the plasmolyzed cell follow the van't Hoff-Boyle law in a fashion analogous to those obtained from a protoplast (12).

The optical method, however, appears to be influenced by other factors which demand caution in interpretation and require verification with independent methods on different systems. Thus, the small optical effect exhibited by R. mooseri cells in purified suspensions when exposed to salts

### Table 5. Intracellular K⁺ and Na⁺ in Rickettsia mooseri

<table>
<thead>
<tr>
<th>Exp. no.</th>
<th>Extracellular K⁺ (meq/liter)</th>
<th>Intracellular K⁺ (meq/liter)</th>
<th>Extracellular Na⁺ (meq/liter)</th>
<th>Intracellular Na⁺ (meq/liter)</th>
<th>Osmolarity</th>
<th>Q₀s (mg of protein)</th>
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<td>15</td>
<td>116</td>
<td>118</td>
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</tr>
</tbody>
</table>

* Flask contents: R. mooseri cells; protein, 8.7 mg in experiment 4 and 4.0 mg in experiment 5; wet weight, 31 mg in experiment 4 and 17 mg in experiment 5; toxin titration, 960 in experiment 4 and 760 in experiment 5. KCl and NaCl as needed to give K⁺ and Na⁺ concentrations shown. K₂HPO₄-KH₂PO₄ (4:1), 1.2 × 10⁻² M with respect to P; Tris buffer (pH 7.4), 1.4 × 10⁻¹ M; K glutamate, 2.5 × 10⁻¹ M; MnCl₂, 10⁻¹ M; MgCl₂, 5 × 10⁻¹ M; ATP, 3 × 10⁻¹ M. Sucrose as needed to give desired osmolality. Total volume, 3.0 ml. Incubation, 3 hr at 37 C.

* High osmolality due to KCl rather than sucrose.
is probably not a reflection of plasmolysis, because the occurrence of this phenomenon with salts was not supported by either the phase-contrast observations or by the isotopic procedure. In addition, the change in optical density was a stable value over a long time interval, and this would not seem to represent an intermediate value observed during a deplasmolysis. Additional support for this view is seen in the studies of Henneman and Umbreit (13) on Na\(^+\) and K\(^+\) permeability of E. coli. They observed a decrease in optical effect over a 30-min period when E. coli was exposed to 0.5 M KCl, from an initial value of 0.30 to a fairly stable value of 0.18, and phase-contrast observations indicated that the cells were no longer plasmolyzed at the end of this period. Summing up the visual, isotopic, and optical-density evidence, it seems probable that the salts quickly penetrated the purified rickettsial cells and perhaps changed the refractive properties of the cytoplasm by some unknown mechanism, possibly related to a salting-out effect. In E. coli, where the visual and isotopic data indicated an impermeability to passive diffusion of KCl and NaCl compatible with that previously reported (20), the optical effects of the various salts reasonably approximated those produced by sucrose or glucose at equal osmolalities.

The data obtained from the measurement of the intracellular K\(^+\) and Na\(^+\) by flame photometry agree with the plasmolysis experiments. Whereas the E. coli cells could control within certain limits their intracellular K\(^+\) and Na\(^+\) levels, the R. moosleri cells, which had been previously frozen, did not have this capability.

Assuming the validity of the experimental methods used in the quantitative measurement of rickettsial permeability, there is a general agreement with visual, phase-contrast studies. The conclusions derived from these combined studies are as follows. (i) R. mooseri appears to possess an osmotically active, functional, and probably structural membrane which is distinct from the cell wall and is located at the surface of a structure analogous to the bacterial protoplast. (ii) The cytoplasmic membrane of R. mooseri in cells which had neither been frozen nor subjected to a purification procedure exhibited passive permeability properties similar to certain bacteria, such as E. coli; i.e., they would plasmolyze in the presence of high osmolalities of both inorganic electrolytes and sucrose. (iii) However, when the rickettsiae had been subjected to a purification procedure or had undergone a freeze-thaw cycle, they incurred some degree of cellular damage which caused increased permeability to electrolytes and the loss in ability to control the intracellular level of these substances. (iv) The freeze-thaw damage to the rickettsial cells was not severe enough to make them passively permeable to certain organic compounds. In earlier studies (14), however, repeated freeze-thaw cycles did result eventually in a marked increase in permeability to reactants in transaminase studies.

The experimental design employed here to demonstrate passive impermeability is not well suited to the demonstration of active specific membrane transport, but it would appear logical to expect the rickettsiae to possess some membrane-transport mechanisms to overcome the observed passive impermeability.

Previous studies of rickettsial metabolism where cell permeability factors seem to be involved should be reinterpreted in light of the present findings.

ACKNOWLEDGMENTS

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