The Isolation of Envelopes of *Escherichia coli* Spheroplasts in the Zonal Ultracentrifuge

(Received for publication, August 30, 1968)

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SUMMARY

Relatively large amounts of cell envelopes from *Escherichia coli* spheroplasts have been isolated in the zonal ultracentrifuge after deoxyribonuclease treatment of the disrupted spheroplasts. The Anderson B-IV zonal rotor was adapted to this isolation. After isopycnic zonal centrifugation through a 34 to 77% sucrose density gradient, the center of the zone containing the cell wall-cell membrane material positions at 58.9% (w/v) sucrose, corresponding to a density of 1.220 g per ml. Such material was further purified by a washing procedure. Electron micrographs indicate that very little material other than envelopes is present in this fraction. The preparation contained fewer than 0.1% intact spheroplasts.

The cell envelope fraction contains 6.6 ± 0.3% of the protein, 0.5 ± 0.2% of the DNA, 2.7 ± 0.3% of the RNA, and 92 ± 2% of the phospholipid of the cell. Variable amounts of putrescine and spermidine are found in this fraction. The content of spermidine was particularly high, amounting to 4 to 10% of the cell spermidine. However, the spermidine on the envelopes was shown to be exchangeable during isolation and was not shown to have been a characteristic membrane component.

Most of the receptor sites for bacteriophage T6 seem to have been retained on the isolated envelopes. These envelopes contain less than 4% of the diaminopimelic acid of the cell, indicating that most of the material of the rigid layer had been removed.

Bacterial membranes have been implicated in numerous metabolic functions, including protein synthesis (1, 2), replication of DNA (3, 4), transport (5, 6), and electron transport and oxidative phosphorylation (7, 8). In connection with studies in this laboratory on the multiplication of the chromosomes of DNA viruses and of bacteria, we wished to characterize the site of biosynthesis of these organelles in *Escherichia coli*, i.e. the site in or on the bacterial membrane at which the DNA is presumed to be associated and duplicated.

The cytoplasmic envelopes of gram-negative bacteria are less well characterized than the membranes and walls of gram-positive organisms. Spheroplasts of gram-negative organisms contain two readily distinguishable layers surrounding the cytoplasm (8, 9). The rigid layer of the wall of the intact bacterium may be removed to a considerable extent by the action of lysozyme, leaving an outer layer of the wall and a cytoplasmic membrane (10, 11). When the bacterial envelopes are isolated following disruption of the spheroplasts, they routinely consist of double layered structures (12, 9). At this time, techniques are not available for separating the two layers cleanly from each other.

The production of membranes by osmotic lysis of spheroplasts, and their separation in a sucrose gradient appeared to be a method potentially useful for our aim, because the entire procedure could be performed at an ionic strength low enough to minimize the dissociation of structures containing nucleic acid and various structurally significant cations. However, it is not possible to handle the large amounts of material desirable for chemical studies by density gradient centrifugation of spheroplast envelopes in conventional swinging bucket rotors. Therefore, until the present time the preparation of membranes derived from osmotically lysed spheroplasts by this method has permitted relatively limited experimentation, such as electron microscopic examination. To isolate larger amounts of envelopes, as in some recent studies (12), the membranes have been separated by centrifugation to form a pellet and the pellet has been washed. It appeared to us that such a procedure might tend to entrap constituents not normally associated with the membranes within the pelleted membranes.

In the present study we report the use of the Anderson B-IV zonal rotor (13) in the development of methods for the isolation and characterization of large quantities of purified envelopes from *E. coli* spheroplasts. This instrument permits a very considerable increase (about 100-fold) in the amount of material isolable by density gradient centrifugation in swinging bucket rotors. It was discovered very early in our experiments that the presence of relatively large molecular weight DNA in suspensions of osmotically lysed spheroplasts affected the separation
of the bacterial envelopes. For the initial orienting studies reported here, therefore, we have degraded DNA with DNase and have isolated and characterized envelopes devoid of significant amounts of the bacterial chromosome. These envelopes have been characterized according to the physical parameters of density and sedimentation rate. We have also determined some of the chemical parameters of the purified envelope fraction, including the contents of lipid phosphorus, protein, nucleic acids, and the polyamines, putrescine and spermidine. Our data indicate that the polyamines which were found on the envelope have been redistributed during isolation.

**EXPERIMENTAL PROCEDURE**

**Methods**

*Bacteria—*E. coli* strain THU was used in these experiments. Strain THU is a polyauxotrophic mutant of strain 15 requiring thymine, histidine, and uracil for growth (14). The concentrations of nutrients used per ml of a high phosphate medium described previously (14) were 1 mg of glucose, 10 μg of thymine, 20 μg of histidine, and 10 μg of uracil. With vigorous aeration in this medium the cells had a doubling time of 72 min.

For growth of cells containing ¹⁴C-spermidine, quantities of ¹⁴C-spermidine up to 2.0 μmoles (1.6 x 10⁶ cpm) were added to 5 min of incubation at 23°, lysozyme was added to a concentration of 10 pg per ml, and EDTA was added to 0.001 M. With vigorous aeration in this medium the cells had a doubling time of 72 min.

Preparation of Lysate—*E. coli* THU was harvested in the exponential phase at 4 x 10⁸ cells per ml by centrifuging the cells at 12,000 x g for 5 min in the cold. The pellets were washed once with 0.01 M Tris chloride pH 8.0, and resuspended at 10⁷ cells per ml in 20% sucrose-0.03 M Tris chloride pH 8.0. Spheroplasts were formed by the method of Neu and Heppel (15); after 5 min of incubation at 23°, lysozyme was added to a concentration of 10 µg per ml, and EDTA was added to 0.001 M. After gentle swirling for 30 min the spheroplasts were centrifuged at 13,000 x g for 10 min, and the pellet was resuspended in 1/3 volume of water.

The spheroplasts were resuspended in water with the aid of a wide bore pipette and swirled gently for 5 min, during which time lysis occurred. The lysate was further incubated with 20 μg of DNase per ml for 15 min at 23°. This treatment resulted in partial degradation of the DNA in the lysate, yielding a solution of low viscosity. Under these conditions the viable count of the lysate was reduced to approximately 0.2% of the untreated cells. In one experiment cells were lysed by the method of freezing and thawing the cells in the presence of 1 mg of lysozyme per ml (16). Lysates prepared in this way also had 0.2% survivors by viable count.

Zonal Centrifugation of Bacterial Lysates—For large scale separation of the cell envelope fraction in the B-IV zonal rotor, 1475 ml of a 34 to 77% sucrose density gradient was formed at 3000 rpm with a peristaltic pump, model 600-1200, Harvard Apparatus Company (Bedford, Massachusetts). The gradient was formed according to the method of Davis, Santen, and Agranoff (17). The sample was centrifuged for 6 x 10⁸ g-min at 5° for isopycnic banding of cell envelope material. The gradient was removed from the rotor at 3000 rpm by pumping 85% sucrose into the rotor, thus forcing the gradient out through the central orifice. The material was usually collected in 10-ml fractions. Each fraction was thoroughly mixed, and the absorbance at 280 mg and the refractive index of each fraction were read. Particulate material was collected by diluting with an equal volume of water and centrifuging at 30,000 x g for 45 min. The pellets were homogenized in the presence of water and recentrifuged; this washing procedure was repeated. A flow sheet of the procedure is shown in Table I.

Chemical Estimations—Lipid was extracted from the cells by the method of Folch, Lees, and Sloane Stanley (18), and the lipid phosphorus was determined by the method of Bartlett (19). Protein was estimated by the procedure of Lowry et al. (20). Nucleic acids were extracted as cold perchloric acid-insoluble, hot acid-soluble material; RNA of the extract was estimated by a modification of the orcinol procedure of Bial as given by Ashwell (21) and DNA by the Burton diphenylamine method (22).

The polyamines were extracted and estimated by the method of Raina and Cohen (23). Samples containing sucrose from the gradient were subjected to additional treatment. After extracting the perchloric acid-soluble material and removing potassium perchlorate, 5 g, wet weight, of acid-washed Dowex 50-H⁺ were added to every 100 ml of the extract at pH 2. The mixture was stirred for 20 min at room temperature and transferred to a column. To the eluate were added an additional 5 g of Dowex 50-H⁺, it was then mixed for 20 min, and the mixture was added to the column. The eluate was discarded. Then 200 ml of 2 N HCl were used to elute the polyamines from the column. The eluate was evaporated to a small volume and brought up to 7.0 ml with water. This solution was then mixed with butanol, and the assay was continued as described by Raina and Cohen. Activity of the succinate oxidase system was estimated by the spectrophotometric method of Elks (24).

Radioisotope counting was carried out on filter papers in a toluene liquifluor system with a Packard Tri-Carb scintillation counter.

Diaminopimelic acid was determined as described by Meadows and Work (25).

Electron Microscopy—Samples were suspended for 10 min in 2% osmium tetroxide in a buffered salt solution, pH 7.9 (26). After centrifugation, the pellets were fixed in the same solution for 2 hours at 2°. The preparations were then post-fixed with 0.5% uranyl acetate in the above buffer at pH 6.0 for 1 hour at room temperature. The specimens were dehydrated in a graded concentration series of ethyl alcohol and embedded in Araldite (International Chemical and Nuclear Corporation, City of Industry, California).

Ultrathin sections were cut with a Porter-Blum ultra-microtome MT-2. Sections were stained with lead citrate (27) and examined in a Siemens Elmiskop Ia electron microscope at 80 kv.

**Materials**

Carrier-free ³²P-inorganic orthophosphate was obtained from E. R. Squibb and Sons, New York. ¹⁴C-Spermidine trihydrochloride (aminopropyltetramethylene-1,4-¹⁴C-diamine·3HCl) and putrescine-1,4-¹⁴C dihydrochloride were obtained from New England Nuclear. Ribonuclease-free deoxyribonuclease

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¹ All sucrose concentrations are expressed as percentage weight per volume; 20% sucrose refers to 20 g of sucrose brought up to 100 ml with water.

² We are grateful to Dr. Margit M. K. Nass and Mr. John W. R. Hobbs for obtaining these data.
TABLE I

**Flow sheet for preparation of envelope fraction**

<table>
<thead>
<tr>
<th>E. coli THU at $4 \times 10^9$ cells per ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>$12,000 \times g$ for 5 min</td>
</tr>
</tbody>
</table>

**Supernatant**

- **Cell pellet**
  - Wash with 0.01 M Tris, pH 8.0
  - Centrifuge at $12,000 \times g$ for 5 min

**Supernatant**

- **Cell pellet**
  - Resuspend at $10^9$ cells per ml in 18.6% sucrose-0.03 M Tris, pH 8.0
  - Add EDTA to 0.001 M and lysozyme to 10 mg per ml
  - Incubate for 30 min at 23°C
  - **Spheroplasts**
    - Centrifuge at $13,000 \times g$ for 10 min

**Supernatant**

- **Spheroplast pellet**
  - Resuspend at 6-fold concentration in water for 10 min with gentle agitation

**Lysate**

- Add DNase; incubate 15 min at 23°C
- Layer onto 34 to 77% sucrose gradient
- Centrifuge for $6 \times 10^6 \text{ g-mins}$
- Collect fractions, read absorbance at 290 nm and refractive indices

**Envelope fraction**

- Dilute with an equal volume of water
- Centrifuge at $30,000 \times g$ for 45 min

**Supernatant**

- **Envelope pellet**
  - Homogenize with water
  - Centrifuge at $30,000 \times g$ for 45 min

**Supernatant**

- **Envelope pellet**
  - Homogenize with water
  - Centrifuge at $30,000 \times g$ for 45 min

**Supernatant**

- **Washed envelope pellet**

(DNase) and crystalline ribonuclease (RNase) were purchased from Worthington. The ribonuclease was heated at 90°C for 10 min prior to use. Egg white lysozyme was obtained from Calbiochem.

Baker analyzed reagent grade sucrose was used for the gradient work. All sucrose solutions were prepared shortly before use and cold-sterilized by Millipore filtration through 0.45 µm pore size filters (Millipore Corporation, Bedford, Massachusetts).

**RESULTS**

We desired a gradient in which the band containing the membranous materials would be well resolved from other cell particulates in an environment of low ionic strength. Such a gradient was 34 to 77% sucrose. Under the conditions of our centrifugation whole cells and unlysed spheroplasts passed through the gradient, but the fraction which contained the cell envelope material was held in the gradient. Ribosomes did not move out from the sample zone.

The physical characteristics of the membrane fraction separated on a linear 34 to 77% sucrose density gradient were determined in an SW-39 rotor (Spinco). A cell lysate, prepared as described above, was separated into portions, each of which was subjected to a different amount of g-minutes. Fractions of the gradients were collected, and the absorbance at 290 nm and refractive indices were read. The position of the absorbance peak associated with envelope material was recorded as a function of the g-minutes applied to the gradient. Conditions of rate-zonal sedimentation prevailed until approximately $6 \times 10^6$ g-min; above this value the position of the center of the band did not change appreciably over a 20-fold range of g-minutes. Under conditions of isopycnic banding, the center of the band was in a region of the gradient corresponding to a sucrose density of 1.220 g per ml, at 58.9% sucrose.

The position of the center of the band as a function of the g-minutes during the rate zonal sedimentation was used to approximate the $s_{20,w}$ of the band, according to the method of...
Shumaker and Rosenblum (28). We made the assumption that diffusion of the envelope material was so small that the diffusion term could be ignored. Equations estimating the viscosity and density of sucrose at 0° were taken from Barber (29). $g_{m,w}$ was estimated to be 2300 ± 200 S.

This band contained 92% of the lipid phosphorus and most of the particulate succinate dehydrogenase activity of the cell; further characterization of this material is given below.

Zonal ultracentrifugation in the B-IV rotor was carried out for $6 \times 10^6 g\cdot min$. This value of $g\cdot min$ is sufficient to move the center of the band almost to an isopycnic position. Less than $6 \times 10^6 g\cdot min$ resulted in rate-zonal sedimentation. Under these conditions the envelope material moved out into the gradient but would not pass through it, as would cells and unlysed spheroplasts. Thus, the gradient was designed such that only the band which included the membranous material and absorbed at 280 nm would position in the center of the gradient. The sedimentation rate and isopycnic position of the center of the envelope band were the same as with swinging bucket runs. A 280 nm absorbance profile is shown in Fig. 1.

Some Chemical Characteristics of Envelope Fraction—Isolated cell envelopes were obtained as in Table I. The gradient fractions were divided into a sample region (I), three middle regions (II), an envelope region (III), and the end region (IV) as shown in Fig. 1. Washed fractions were analyzed for DNA, RNA, protein, and lipid phosphorus, and the results of these experiments are shown in Table II.

The zone in which the sample was layered (Region I) contained almost all of the DNA that resisted DNase. This sample zone also contained 87% of the protein and 98% of the RNA of the cell; in this region were included soluble protein and RNA as well as ribosomal material.

In an effort to have an environment of low ionic strength, no Mg++ was added to the lysate. Because of the low Mg++ concentration in the lysate, the DNase treatment was only slowly effective. Thus, under the conditions described in this paper, 34% of the DNA of the cell was still recovered from the lysate, the DNase treatment was only slowly effective. Only 93% of the protein of the cell was recovered in the lysate. The remaining 7% was released into the spheroplasting medium (buffer used when forming spheroplasts) upon treatment with EDTA and lysozyme (15).

The fractions from Region II of the gradient contained only small quantities of the compounds tested, as can be seen in Table II. The end region (IV) of the gradient was also low in these compounds. The fraction including envelopes (Region III) contained significant amounts of RNA, protein, and lipid phosphorus, and was studied further.

Electron photomicrographs are given in Fig. 2 for whole cells (a), spheroplasts (b), envelopes taken directly from the gradient (c), and envelopes after extensive washing (d). The envelopes appear as vesicles of a size similar to unlysed spheroplasts. The double layered structures surrounding the spheroplasts are evident in the envelope fractions. Almost every vesicle seems to have two layers, one very electron-dense and distinct, and one less electron-dense and less distinct. Presumably the very dense layer of the isolated envelope corresponds to the outermost layer of the spheroplast. There seems to have been no considerable inversion of the vesicle layers. The photomicrographs of the washed envelope material show few recognizable structures other than envelopes. The preparations of envelopes contained less than 0.1% intact spheroplasts.

In Table III are shown data from eight experiments on the

![Fig. 1. Absorbance at 280 nm of a lysate fractionated on a 34 to 77% sucrose density gradient for 6 $\times$ 10^6 g·min in the B-IV rotor.](image)

**Table II**  
*Analysis of preparation described in Fig. 1*

<table>
<thead>
<tr>
<th>Sample</th>
<th>DNA phosphorus*</th>
<th>RNA phosphorus*</th>
<th>Protein</th>
<th>Lipid phosphorus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pmol/cell X 10^9</td>
<td>pmol/cell X 10^9</td>
<td>µg/cell X 10^9</td>
<td>%</td>
</tr>
<tr>
<td>Initial culture</td>
<td>34.1</td>
<td>100</td>
<td>172</td>
<td>100</td>
</tr>
<tr>
<td>Lysate*</td>
<td>13.2</td>
<td>34</td>
<td>171</td>
<td>99</td>
</tr>
<tr>
<td>Region I, sample zone</td>
<td>4.8</td>
<td>14</td>
<td>109</td>
<td>98</td>
</tr>
<tr>
<td>Region II, middle zones</td>
<td>0.20</td>
<td>0.6</td>
<td>0.125</td>
<td>0.07</td>
</tr>
<tr>
<td>Region III, membrane zone</td>
<td>0.20</td>
<td>0.6</td>
<td>4.31</td>
<td>2.5</td>
</tr>
<tr>
<td>Region IV, end zone</td>
<td>0.03</td>
<td>0.1</td>
<td>0.125</td>
<td>0.08</td>
</tr>
</tbody>
</table>

* Acid-precipitable

* Lysate of EDTA- and lysozyme-treated cells incubated in the presence of 20 µg of DNase per ml.
FIG. 2. Electron photomicrographs of cells, spheroplasts, and envelope fractions. Envelope fractions were isolated as described under "Experimental Procedure." Samples of cells (a) and spheroplasts (b) were taken, as well as samples of envelopes directly from the gradient (c), and envelopes after thorough washing (d). All preparations were fixed with 2% osmium tetroxide and post-fixed with 0.5% uranyl acetate. Magnification × 18,000.
chemical characteristics of the cell, cell lysate, and envelope fraction. The fraction containing the cell envelope that results from lysis of lysozyme- and EDTA-treated spheroplasts contained 92% of the lipid phosphorus of the cell and 6.6% of the protein of the cell. This fraction, which was centrifuged from a DNase-treated lysate, contained 0.5% of the DNA of the cell. However, 2.7% of the RNA of the cell was found to remain with the membrane fraction, even after extensive washing and re-centrifugation of the material obtained by density gradient centrifugation. The average values for DNA, RNA, and protein had small standard deviations.

The fractions were also assayed for their content of putrescine and spermidine (Table III). There was no detectable polyamine in the gradient between the sample zone and the membrane band (Region II) or between the membrane band and the rotor wall (Region IV). The putrescine content of the membrane fraction was found to be 1.1% of the total putrescine of the cell, with a range of 0.7 to 1.5%. Varying amounts of spermidine were found within this band, ranging from 2.1 to 10%, with an average of 6.0% of the spermidine of the cell. The large variation in the amount of spermidine within the envelope fraction does not appear to be due simply to the presence of EDTA in the lysing procedure. The envelope fraction from one-half of a batch of cells was obtained as described under “Methods.” The other half of the cells were lysed by freezing and thawing three times in the presence of lysozyme, and after DNase treatment the envelopes were also separated from this lysate in the zonal rotor. The absence of EDTA from this latter lysis mixture did not produce significant differences in analytical values.

The putrescine nitrogen and the spermidine nitrogen available to balance negative charges on the membrane have been totaled for each experiment in Table III. The ratio of RNA phosphorus to polyamine nitrogen varies only over a limited range. In all experiments the ratio of RNA phosphorus to polyamine nitrogen varied only over a limited range. In all experiments the ratio of RNA phosphorus to polyamine nitrogen varied only over a limited range. In all experiments the ratio of RNA phosphorus to polyamine nitrogen varied only over a limited range. In all experiments the ratio of RNA phosphorus to polyamine nitrogen varied only over a limited range. In all experiments the ratio of RNA phosphorus to polyamine nitrogen varied only over a limited range.

In some of the experiments listed in Table III, cells were grown in the presence of 14C-spermidine at concentrations below 0.25 μM or less. The spermidine was taken up into the cell within 20 min after addition and remained in the cell for at least two generations. The distribution of the radioactive spermidine on the membrane was similar to the distribution of endogenous spermidine on the membrane. The specific activities of spermidine in five different experiments were indistinguishable for cells and for envelopes.

The presence of significant amounts of spermidine on the envelope was of interest because polyamines have been shown to affect lysis of membranous organelles, such as mitochondria. We were concerned, therefore, with the possible association of spermidine with phospholipids, as well as with the possibility that the spermidine found had been adsorbed after cell disruption.

**Polyamines and Envelope Lipid**—We have determined the polyamine content of the lipid fraction extracted from envelope material. The envelope fraction was isolated from cells which had been grown in the presence of 14C-spermidine. After the envelope fraction had been lyophilized, the lipid fraction was extracted by the technique of Polch et al. (18), and the distribution of the radioactive spermidine was monitored throughout the procedure. Most of the polyamine in the envelope fraction was not solubilized with the lipid material, but was found in the insoluble fraction containing protein and RNA. Of the less than 3% of the total polyamine on the envelope extracted into chloroform-methanol, almost two-thirds resisted extraction into water. Only 1.7% of the envelope polyamine is left in the lipid fraction after this aqueous wash.

**Exchange of “Envelope” Polyamine with Soluble Polyamine**—Cells were grown in the presence of 14C-spermidine, lysed as described, and the lysate was centrifuged at 30,000 × g for 45 min to pellet the crude envelope fraction. The resulting supernatant, containing labeled polyamine, was used to lyse spheroplasts prepared from cells grown in the absence of label. The envelope fraction of the unlabeled cells was then collected in the zonal centrifuge and washed. This membrane fraction was found to have a specific activity of spermidine close to that value predicted if total equilibration between envelope polyamine and the polyamine in the lysis solution had occurred (Table IV).

Lysis with a supernatant fraction of a lysate or with water resulted in membranes containing comparable amounts of polyamine. Lysis of spheroplasts with such a supernatant resulted in 0.3% survivors by viable count.

**Effects of DNase and RNase Treatment on Distribution of Polyamine**—In all of the above experiments DNase was added to the lysate prior to zonal centrifugation. The studies presented below, in which no DNase was added, were not carried out in the B-IV zonal rotor because DNA was distributed somewhat unevenly throughout the gradient. This apparently resulted from fragmentation of the DNA upon passage of the sample through the small orifice. Instead, envelopes were only crudely separated from lysates by centrifugation at 30,000 × g for 45 min.

Lysates were prepared as described above except that DNase was not added immediately. The lysates were separated into five batches: a control (no enzymes added), one to which RNase was added, one to which RNase was added followed by DNase addition, one to which DNase was added, and one to which DNase was added followed by RNase addition. Incubation of
stirred vigorously, and separated into five groups for treatment.

The procedures have been designed to subject the cell fractions to an environment of low ionic strength since the distribution of some charged compounds has been investigated. The gradient is composed of unbuffered sucrose; the only ions added to the gradients are those contributed by the cell contents. We have investigated some of the chemical and physical characteristics of the envelope even though both wall and membrane components are present. Of the material in the rigid layer, less than 4% remains, judging from the diaminopimelic acid content of the spheroplasts. A high degree of purity of the envelope fraction has been attained, with collection of relatively large quantities of material. This rotor has been used to isolate envelopes from 2 to 10 liters of culture at 4 x 10^8 cells per ml; the resultant envelope fraction has the same characteristics as envelopes isolated from 6 to 20 ml of culture with the SW-39 rotor. We could have scaled up to even larger amounts of sample, since the resolution of the envelope band from the sample zone would not be endangered by mild band spreading. The gradient is designed to trap only the envelope material; ribosomes do not move out into the gradient, and intact cells and spheroplasts pass all the way through the gradient.

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**Table IV**

*Exchange of ^14C-spermidine during isolation of envelopes*

*E. coli* THU was grown in two 2-liter batches until 4 x 10^8 cells per ml were attained. Cells from one flask were grown in the presence of ^14C-spermidine to a cellular specific activity of 640 cpm per mumole. A lysate was prepared as described, and the envelope fraction was removed by centrifugation at 30,000 x g for 45 min. One-half the supernatant from this centrifugation was used to lyse spheroplasts prepared from the other batch of cells, grown in the absence of radioisotope. The envelopes from the unlabeled cells were then isolated by sucrose density gradient centrifugation and washed as described. Specific activities of the spermidine of the fractions were obtained.

<table>
<thead>
<tr>
<th>Envelope-free lysate from ^14C-spermidine-labeled cells</th>
<th>Total spermidine</th>
<th>^14C-Spermidine</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmoles</td>
<td>mmole</td>
<td>cpm</td>
<td>cpm/mumole</td>
</tr>
<tr>
<td>2680</td>
<td>1496</td>
<td>9.0 x 10^4</td>
<td>640</td>
</tr>
</tbody>
</table>

Unlabeled spheroplasts

Lysate of unlabeled spheroplasts lysed in presence of ^14C-spermidine-labeled envelope-free lysate

<table>
<thead>
<tr>
<th>Sample zone, Region I, Fig. 1</th>
<th>Total spermidine</th>
<th>^14C-Spermidine</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmoles</td>
<td>mmole</td>
<td>cpm</td>
<td>cpm/mumole</td>
</tr>
<tr>
<td>6880</td>
<td>3120</td>
<td>8.6 x 10^4</td>
<td>186</td>
</tr>
</tbody>
</table>

Expected value if total equilibrium of ^14C-spermidine with ^14C-spermidine on envelope

<table>
<thead>
<tr>
<th>Expected value</th>
<th>Total spermidine</th>
<th>^14C-Spermidine</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>nmoles</td>
<td>mmole</td>
<td>cpm</td>
<td>cpm/mumole</td>
</tr>
<tr>
<td>230</td>
<td>242</td>
<td>4.2 x 10^4</td>
<td>172</td>
</tr>
</tbody>
</table>

The washed envelope fraction adsorbs bacteriophage T6 at 82% of the rate of the intact cells. The velocity constants were determined as described by Adams (30). Envelopes derived from 1 x 10^8 cells per ml were used to adsorb phage the initial concentration of which was 1.6 x 10^9 particles per ml. The plot of log P_9/P versus time resulted in a straight line down to 20% adsorption, the lowest point investigated. Most of the receptor sites for T6 still seemed to be available to the phage and had therefore been retained on the isolated envelopes.

**Discussion**

The Anderson B-IV zonal rotor has been used to isolate cell envelopes of *E. coli* prepared from lysozyme- and EDTA-treated spheroplasts. A high degree of purity of the envelope fraction has been attained, with collection of relatively large quantities of material. This rotor has been used to isolate envelopes from 2 to 10 liters of culture at 4 x 10^8 cells per ml; the resultant envelope fraction has the same characteristics as envelopes isolated from 6 to 20 ml of culture with the SW-39 rotor. We could have scaled up to even larger amounts of sample, since the resolution of the envelope band from the sample zone would not be endangered by mild band spreading. The gradient is designed to trap only the envelope material; ribosomes do not move out into the gradient, and intact cells and spheroplasts pass all the way through the gradient.

The procedures have been designed to subject the cell fractions to an environment of low ionic strength since the distribution of some charged compounds has been investigated. The gradient is composed of unbuffered sucrose; the only ions added to the gradients are those contributed by the cell contents. We have investigated some of the chemical and physical characteristics of the envelope even though both wall and membrane components are present. Of the material in the rigid layer, less than 4% remains, judging from the diaminopimelic acid in the envelope fraction. Kaback and Stadtman reported
that 10% or less of the diaminopimelic acid of the cell wall could be found in membranes of E. coli prepared from penicillin spheroplasts (12).

The density of the material in the envelope fraction is higher than would be expected for a pure lipoprotein membrane fraction, indicating the presence of dense material (probably carbohydrate) in the fraction. Phage receptor sites are present and available to the phage. Since the receptor site for T6 resides in the outer lipoprotein wall preparations (8), our envelopes contain wall as well as membrane components. The electron photos show the presence of two kinds of electron-dense layers; each vesicle seems to have one of each kind. The presence of double layered structures is typical of collapsed spheroplast envelopes (9). Comparison of intact spheroplasts with the purified envelopes indicates that two membranes present in each spheroplast are carried together in a purified vesicle. It is interesting that the washed vesicles approach the same size as the intact spheroplast; apparently the lysis is gentle enough to burst the spheroplasts without destroying the size of the envelopes. It seems unlikely that a reaggregation of small pieces to this precise size and shape takes place.

We have found that the envelope fraction contains 92% of the lipid, 6.6% of the protein, 0.5% of the DNA, and 2.7% of the RNA of the cell. All of these average values have small standard deviations compared to those of the polyamines, especially spermidine. These values did not change upon further washing of the envelopes and are not dissimilar from values reported by several workers for different preparations of E. coli membranes (12, 31, 32). Our values for RNA are consistently lower than those reported by these other workers, most of whom used differential centrifugation techniques with small quantities of material.

It is difficult at this time to speculate on a possible physiological role (or roles) of this RNA on the envelope. Sutl demonstrated that after a short pulse of 32P04 was given to E. coli cells or spheroplasts, the RNA of highest specific activity in subcellular fractions was found in the membrane fraction (31). The implication of this rapidly labeled RNA could be messenger RNA on the membrane; however, ribosomes or ribosomal RNA (or both) might have been present in the membrane fractions obtained in the studies cited above as well as in our preparations. Indeed, the membrane-cell wall fraction of E. coli disrupted with a Hughes press and separated by differential centrifugation has been shown to contain RNA with a base composition similar to ribosomal RNA (31).

Partial degradation of DNA with DNase and extensive washing of the membrane fraction did not remove all of the DNA from the membrane; 1.54 × 10−19 mole of DNA phosphorus per cell remained with the envelope. If the membrane is the site of replication (4), then perhaps this remaining DNA will turn out to include the replicating region. This paper provides a method for the collection of large amounts of envelopes so that some of the implications of the presence of nucleic acid on a membrane may be investigated, providing that its presence is not an artifact of isolation.

In contrast to the fact that there is low variation in the nucleic acid data, the values for the polyamine content of the envelope fraction, especially those of spermidine, show a high degree of variation. The envelopes are capable of holding putrescine and spermidine through three aqueous washes, each followed by homogenization with a Dounce homogenizer. We have not demonstrated the existence in vivo of envelope-bound spermidine. The mixing experiment (Table IV) indicates that exogenously supplied 14C-spermidine can equilibrate with the endogenous 14C-spermidine, including that fraction ultimately detected on the envelope. This equilibration might occur within the soluble pool and then portions of the equilibrated spermidine pool might add onto the envelope during isolation. It is also possible that there might be spermidine on the envelope available for exchange with exogenously supplied 14C-spermidine.

A significant portion of the spermidine in the lysate seems to be bound to material containing RNA. There is a correlation between the degradation of RNA in the 30,000 × g supernatant and the availability of spermidine to precipitate with the envelope fraction. Most of the RNA that was degraded was probably ribosomal RNA, and in the absence of this RNA the envelope fraction can accept a large share of the spermidine of the cell. No such correlation was established for DNA. Treatment with DNase did not affect the distribution of spermidine, even though the distribution of DNA was altered. Also, when the membrane obtained by differential centrifugation was washed, most of the DNA that was present was washed off, but most of the RNA and spermidine remained.

In all preparations of envelopes described in this work there is sufficient RNA phosphorus within the envelope fraction to account for all the cation provided by polyamine (Table III). It is possible that the RNA is the substance on the envelope responsible for binding of the spermidine. Envelope-bound polyamine is not solubilized by chloroform-methanol, but is found in the insoluble fraction containing protein and nucleic acid. If the polyamine found on the envelope were held there by negatively charged lipid phosphorus, we might expect to see more than 3% of the envelope spermidine solubilized by chloroform-methanol. We cannot exclude the possibility that acidic groups on proteins of the envelope have a role in holding the polyamine to the envelope.

If much of the spermidine of the cell preferentially attaches to RNA rather than to DNA, how could such selective binding occur? One possibility is that there is some compartmentalization of spermidine with RNA, perhaps at the site of synthesis of RNA. In eucaryotic organisms the nucleolus seems to be the site of origin of ribosomal RNA (34, 35). Methylation of the RNA also seems to occur in the nucleolus (35). Since S-adenosylmethionine is present for methylation to occur, and since S-adenosylmethionine is used in the synthesis of spermidine, perhaps the site of synthesis of spermidine is in the nucleolus. If there is an analogous nucleolar region on the membrane of the bacterium, it is conceivable that spermidine and ribosomal RNA are synthesized concomitantly and transferred to the cytoplasm together. If this occurred, preferential attachment of spermidine to RNA over DNA could result.

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