

# Topography of Reaction Center Subunits in the Membrane of the Photosynthetic Bacterium, *Rhodospseudomonas sphaeroides*

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**ABSTRACT** The localization of the reaction center polypeptides (L, M, and H) in the membranes of both the wild-type, strain 2.4.1, and the carotenoidless mutant, R-26, of *Rhodospseudomonas sphaeroides* was determined by using affinity-purified antibodies specific for these proteins. Binding of the antibodies to reaction center subunits in spheroplasts was visualized in the electron microscope by immunoferritin labeling. The H and M subunits were labeled at both the cytoplasmic and the periplasmic surfaces of the membrane, whereas the L subunit was labeled only at the periplasmic surface of the membrane. Thus, the reaction center is asymmetrically oriented in the membrane with at least two subunits (H and M) spanning the membrane.

The plasma membrane of the photosynthetic bacterium, *Rhodospseudomonas sphaeroides*, exhibits an intricate series of invaginations (1, 2) that harbor the reaction center (RC) polypeptide subunits. The RCs have been isolated by detergent extraction (for a review, see, for example, reference 3) and are believed to be integral membrane proteins (4). The subunits, designated L, M, and H, are in a 1:1:1 stoichiometry (5). Their molecular weights, determined from an analysis of their amino acid compositions (6, 7) were found to be 28,000, 32,000, and ~34,000, respectively. Each RC contains four bacteriochlorophylls, two bacteriopheophytins, two ubiquinones, and one iron (3). Together with these cofactors, the RC protein accomplishes the conversion of light into electrochemical energy.

According to the chemiosmotic hypothesis (8), the topographical organization of the membrane components is fundamental to the directional transfer of protons and electrons and to the coupling of these events to the generation of ATP. Thus, localization of the RC subunits with respect to the membrane should aid in determining their function in the primary charge separation which initiates cyclic electron transfer.

Several techniques have been used to investigate the topography of the RC subunits. They include: precipitation with (9), and adsorption of (10), antisera, labeling with antibodies (11–14), radiochemical labeling (15–18), photoaffinity labeling (19), and enzymatic digestions (16, 18, 20). These studies have shown that the RC is an integral membrane protein with the H subunit being exposed on the cytoplasmic side of the membrane. However, no clear consensus has been reached concern-

ing the topography of the other subunits (for a more detailed discussion, see last section of this paper).

We used specific antibodies to probe the topography of RCs in the membrane by indirect immunoferritin labeling (21). In this technique, the photosynthetic membrane is first exposed to rabbit antibodies directed against the RC subunits and then to ferritin-conjugated goat antibodies that bind to rabbit IgG. Ferritin is an electron-dense molecule, thereby permitting localization of the binding site by electron microscopy.

We performed our initial work on chromatophores, which are closed, inverted membrane vesicles purified from disrupted bacteria (10, 22, 23). Antibodies against both H and RC were found to label the outside of chromatophores (12). The inaccessibility of the inside surface of intact chromatophores, together with the difficulty of unambiguously assigning the membrane sidedness of chromatophores, led us to the use of spheroplasts. Their fragility allowed membrane disruption thereby exposing the cytoplasmic membrane surface without, however, altering the basic morphology of the spheroplasts. An important feature of our study was the characterization of the purified antibodies to insure their specificity against their respective subunits. Preliminary reports of this work have appeared (12–14).

## MATERIALS AND METHODS

### *Preparation of H, LM, L, and M*

H and LM form a relatively loose complex that can be dissociated with a chaotropic agent (LiClO<sub>4</sub>). L and M, on the other hand, form a much tighter

complex that can be dissociated only with a strong detergent (SDS). The details of the purification procedure are as follows:

The H subunit was prepared by centrifugation of RCs in a solution containing the chaotropic agent,  $\text{LiClO}_4$ , by a modification of the method described previously (appendix B-1 of reference 3). Reaction centers ( $A_{800}^{1\text{cm}} = 15$ ,  $\sim 0.5$  ml) in 0.1% lauryl dimethyl amine oxide (LDAO) (Onyx Corp., Jersey City, NJ), 50 mM Tris-HCl, pH 8, were layered onto a solution of 1 M  $\text{LiClO}_4$  in the same buffer and centrifuged in a Spinco SW 65 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA) at 60,000 rpm ( $\sim 250,000$  g) for 20 h at 20°C. The top of each tube contained the H-subunit; it was removed and dialyzed against 10 mM Tris-HCl, 0.1% LDAO, 1 mM EDTA, pH 8. The purity of this H preparation, as determined by SDS PAGE was  $\sim 90\%$ . After dialysis, the buffer was made 0.1 M in NaCl. Typically, the preparation contained several mg of H obtained by pooling material from several centrifuge preparations. The sample containing H was passed through a 1.0- $\times$ -4-cm column packed with affinity purified anti-LM IgG covalently coupled to Ultrogel AcA 22 (LKB Instruments, Inc., Rockville, MD) (1 mg IgG/ml gel) which had been equilibrated with the sample buffer. In this step the LM and RC contaminants were removed. The effluent was then passed through a 1.3- $\times$ -8-cm column packed with affinity-purified anti-H IgG covalently coupled to Ultrogel AcA 22 (1 mg IgG/ml gel) and equilibrated with the same buffer. The column was washed to remove unbound pigments and proteins. The bound H was eluted with 0.2 N HCl-glycine, 0.025% LDAO, pH 2.2. The fractions were neutralized with 1 M  $\text{K}_2\text{HPO}_4$ , as they were collected. The affinity purified H was dialyzed at 4°C against 10 mM Tris-HCl, 0.1% LDAO, pH 8, and was concentrated by ultrafiltration. This method produced H with a greatly reduced pigment content. The purity of H, as determined by SDS PAGE, was  $>97\%$ .

The LM complex was prepared as described previously (appendix B-2 to reference 3; 24). An additional purification step was added to remove the residual H and RC contaminants: the LM was passed through a column (1.3- $\times$ -8-cm in 10 mM Tris-HCl, 0.1% LDAO, 0.1 M NaCl, 1 mM EDTA, pH 8) packed with affinity-purified antibodies to H covalently bound to Ultrogel AcA 22 (1 mg IgG/ml gel). The resulting LM preparation contained  $<3\%$  contaminants (determined by SDS PAGE).

The L subunit was prepared from purified LM (see above) as previously described (appendix B-3 in reference 3), except that the LM was concentrated by ultrafiltration. The separation procedure was based on the fact that the L subunit contains a free sulfhydryl group that binds to an affinity resin, whereas the M subunit is devoid of cysteine and does not bind. The binding capacity of the affinity column containing p-hydroxymercuribenzoate (PMB)-derivatized Sepharose 4B was tested with cysteine; it bound 1  $\mu\text{mol}$  of cysteine/ml of Sepharose and  $\sim 1$  mg of L subunit per ml of Sepharose. We carried out all procedures in the dark to prevent photooxidation of amino acids mediated by the pigments present. The contamination with M was  $\leq 3\%$  (determined by SDS PAGE).

The crude M fraction, which passed through the PMB Sepharose 4B column during the preparation of L, was contaminated with  $\sim 10\%$  L. To decrease this contamination, the sulfhydryls of L were reduced with dithiothreitol (Calbiochem-Behring Corp., La Jolla, CA) which was added to a concentration of 1 mg/ml. The sample ( $\sim 30$  ml, containing 30 mg of M) was incubated at 65°C for 30 min and dialyzed overnight at room temperature against 1 l of deoxygenated buffer (100 mM Tris-HCl, 1 mM EDTA, 0.1% SDS, pH 8) and reappplied to a deoxygenated, PMB-derivatized Sepharose 4B column ( $\sim 20$  ml of gel for a 20-mg M sample). The heavily pigmented fraction that passed through unbound contained the purified M subunit. It was contaminated with  $\leq 3\%$  L (determined by SDS PAGE).

### Extinction Coefficients of Isolated L, M, and H Subunits

The concentration of subunits in the sample was obtained by comparing the areas of their SDS PAGE scans with those obtained from RCs. Preparations of each of the isolated subunits were run on SDS PAGE in parallel with RCs. From the integrated areas of the 560 nm scans of the Coomassie-Blue-stained gels of the RCs and the individual subunits, the ratio of concentrations of L, M, and H to RCs was determined. From this ratio and the measured absorbance at 280 nm of the RCs and the subunits that were applied to the gel, the extinction coefficients of L, M, and H were determined by using the known extinction coefficient of RCs ( $\epsilon_{280} = 3.51 \pm 0.16 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$  [3, 25]). The following values were obtained: L:  $\epsilon_{280} = 1.1 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ ; M:  $\epsilon_{280} = 1.9 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ ; and H:  $\epsilon_{280} = 0.46 \times 10^5 \text{ M}^{-1} \text{ cm}^{-1}$ .

### SDS PAGE

We followed the procedure as previously described (5), except for the sample preparation. To  $\sim 1$  nmol of either RCs, LM, L, M, or H distilled  $\text{H}_2\text{O}$  was added to a final volume of 80  $\mu\text{l}$ . Addition of 10  $\mu\text{l}$  of 10% SDS (BDH Chemicals, Ltd.,

Poole, England) and 10  $\mu\text{l}$  of 10% dithiothreitol was followed by heating at 65°C for 45 min. Sucrose (25  $\mu\text{l}$  of 50%) was added and 10–50  $\mu\text{l}$  of the sample was layered on the gel.

### Preparation of Rabbit Antisera and IgG Fractions

Female New Zealand White rabbits were each immunized with 1 mg of protein (RC, LM) emulsified with complete Freund adjuvant (Difco Laboratories, Detroit, MI). After 3 wk they were given booster injections of 1 mg of protein emulsified with the incomplete Freund adjuvant. 3 wk later, intravenous injections of protein (0.2 mg) were administered and the rabbits were bled after 7–10 d to obtain antisera. IgG fractions were obtained from antiserum by ammonium sulfate precipitation and chromatography (26) on diethylaminoethyl-cellulose (Whatman, Inc., Clifton, NJ).

### Preparation of Immunoabsorbent Gels for Affinity Chromatography

The purified RC proteins were coupled to Ultrogel AcA 22 with glutaraldehyde, EM grade (Ted Pella, Inc., Tustin, CA) by a modification of the method of Guesdon and Avrameas (27). The gel was equilibrated with the appropriate buffer: PBS (20 mM sodium phosphate, 150 mM NaCl, pH 7, 0.1% SDS) and 1 mM EDTA, for coupling the L and M proteins; PBS, 0.1% LDAO, 1 mM EDTA, for coupling the RC, LM, and H proteins; PBS, was used when coupling IgG. Sodium azide (0.02%) was added to all solutions as a preservative. The gel was kept suspended in a protein concentration of 20  $\mu\text{M}$  for 48 h. The L and M proteins were incubated at room temperature and RC, LM, H, and IgG were incubated at 4°C. The unbound protein was washed from the gel and monitored optically to determine the amount of bound protein. Typically, 10 nmol of protein were bound per milliliter of gel. Remaining sites of activation in the gel were coupled to L-lysine by mixing an equal volume of 0.1 M L-lysine in 0.1 M sodium phosphate buffer, pH 7, with the coupled gel and incubating the mixture overnight at room temperature for L and M gels and at 4°C for RC, LM, H, and IgG gels. The gel was packed into a column and washed with several column volumes of the incubation buffer followed by two-column volumes of 0.2 N HCl-glycine buffer (prepared by adding 2 M glycine to 0.2 N HCl to obtain pH 2.2). Finally, the gel was washed with one-column volume of 0.2 M  $\text{K}_2\text{HPO}_4$ , followed by several column volumes of PBS (both included 0.025% LDAO for RC, LM, and H). Washing of the L and M gels with 0.2 N HCl-glycine, pH 2.2, resulted in the removal of much of the bound SDS from the proteins. The majority of the pigment associated with the RC proteins was also eluted by the acidic wash. The gels were stored at 4°C.

### Preparation of Antibodies to the H, L, and M Subunits and to Rabbit IgG by Affinity Chromatography

ANTI-H: Rabbit antiserum to RC protein was passed through a 0.45  $\mu\text{m}$  Millipore filter (Millipore Corp., San Francisco, CA) and then through the H-affinity column. The column was washed with PBS, 0.025% LDAO, to remove unbound protein until the absorbance of the effluent,  $A_{280}^{1\text{cm}}$ , was  $<0.05$ . The bound antibodies were eluted with 0.2 N HCl-glycine buffer, 0.025% LDAO, pH 2.2, and neutralized with 1 M  $\text{K}_2\text{HPO}_4$ . The column was washed with one-column volume of 0.2 M  $\text{K}_2\text{HPO}_4$ , 0.025% LDAO, followed by several column volumes of PBS, 0.025% LDAO to regenerate it for further use. The affinity-purified antibodies to H were dialyzed against PBS at 4°C. Affinity-purified antibodies from rabbit antisera have been shown to contain antibodies which are limited to the IgG class (27). Typically, 15 ml of antiserum to RCs were passed through a 5-ml H-affinity column yielding 3 mg of antibodies to H. The affinity-purified antibodies to H contained contaminant antibodies to LM. To eliminate this contamination, the antibodies were passed through a LM-affinity column (see Fig. 1). We determined the specificity of the resultant antibodies by radioimmunoassay techniques. Affinity-purified antibodies were stored at 4°C in PBS, 0.02%  $\text{NaN}_3$ .

ANTI-L AND ANTI-M: Antisera to the LM protein were used to purify antibodies to L and to M in a similar manner, except that the buffers did not contain LDAO. Antiserum to LM was passed through an L-affinity column and unbound protein was washed out with PBS, 0.02%  $\text{NaN}_3$ . The antibodies to L were bound and eluted as described above. After dialysis, any contaminating antibodies to M were removed by passage through a M-affinity column. To reduce further the anti-M contamination, the effluent was passed, adsorbed, and eluted once more through the L-affinity column to obtain twice affinity-purified antibodies to L. A schematic representation of the purification procedure is shown in Fig. 1. The unbound antiserum to LM from the L-affinity column was passed through an M-affinity column and treated in a manner analogous to the

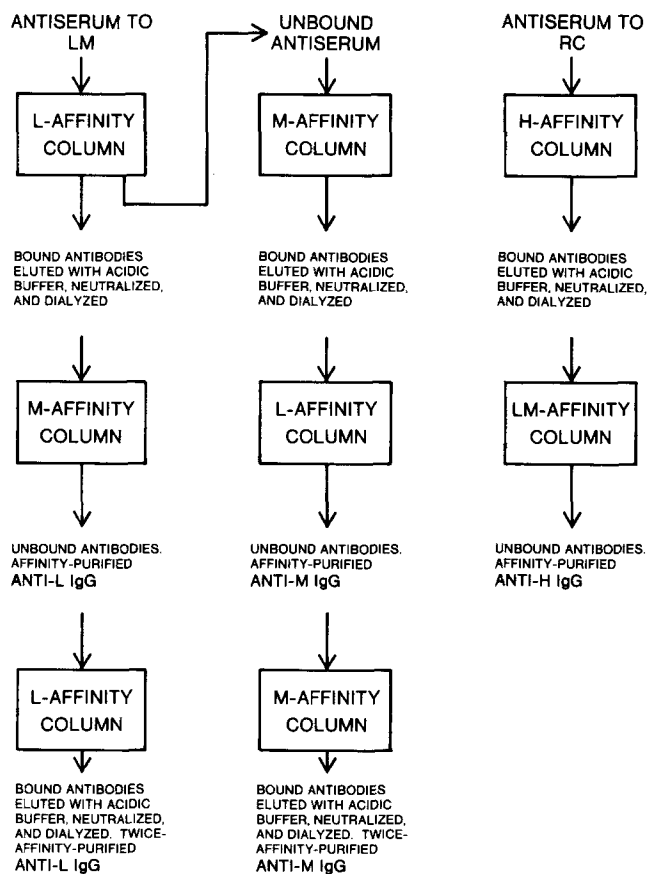


FIGURE 1 Schematic representation of the purification procedure of antibodies against L, M, and H.

one described before to obtain twice affinity-purified antibodies to M (see Fig. 1). The columns were regenerated for further use as previously described. Typically, 20–40 ml of antiserum to LM were passed through a 5-ml L-affinity column followed by a 5-ml M-affinity column. Approximately 2–3 mg of each preparation of affinity-purified antibodies to L and M were recovered.

**GOAT ANTISERUM:** Goat antiserum to normal rabbit IgG (obtained from the laboratory of S. J. Singer, University of California, San Diego, CA) was passed through a normal rabbit IgG-affinity column. Typically, 10 ml of goat antiserum to rabbit IgG was passed over a 30-ml normal rabbit IgG column; ~60 mg of goat antibodies specific for rabbit IgG were affinity-purified.

### Radioiodination of RC Polypeptides

The radioiodination of RC polypeptides was performed with 1,3,4,6-tetrachloro-3 $\alpha$ ,6 $\alpha$ -diphenylglycoluril (Pierce Chemical Co., Rockford, IL). Reaction vessels were prepared by coating borosilicate glass tubes (10  $\times$  75 mm) with 10  $\mu$ g of this reagent as previously described (28, 29). 1 nmol of either RC, LM, or H in 100  $\mu$ l of PBS, 0.1% LDAO, or 2 nmol of L or M in 100  $\mu$ l of PBS, 0.1% SDS, were pipetted into the reaction vessel. The addition of 1 mCi of Na<sup>125</sup>I (carrier-free, ICN, Radioisotope Div., Irvine, CA) initiated the reaction. The reaction vessel was rotated gently for 15 min at room temperature. Addition of 150  $\mu$ l of 0.5 M NaI quenched the radioiodination. The sample was applied to a Sephadex G-50 fine (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, NJ) column (1.3  $\times$  18 cm) equilibrated with PBS, 0.1% LDAO, 0.05% bovine serum albumin (BSA) (Miles Laboratories, Inc., Elkhart, IN), 0.02% NaN<sub>3</sub> for RC, LM, or H, and PBS, 0.1% SDS, 0.05% BSA, 0.02% NaN<sub>3</sub> for L or M. The void volume, containing the radio-labeled protein was collected, divided into 10 aliquots, frozen in liquid nitrogen, and stored at -70°C. The specific activity of the proteins was ~5  $\mu$ Ci/ $\mu$ g.

### Assaying the Binding of Antibodies to <sup>125</sup>I-labeled Antigens

The binding of antibodies to RCs and their subunits was assayed by separate incubations of serial dilutions of the antibodies with each of the <sup>125</sup>I-labeled antigens (RC, LM, L, M, or H) in the dark. Serial twofold dilutions of antibodies

were added together with <sup>125</sup>I-labeled antigen at a concentration of ~5  $\times$  10<sup>-11</sup> M to glass culture tubes (10  $\times$  75 mm) containing 1 ml of incubation buffer (PBS, 0.025% sodium cholate, 0.02% NaN<sub>3</sub>, 0.4% normal rabbit serum for RC, LM, L, or M, and 0.025% LDAO instead of sodium cholate for H). After 48 h of incubation at 4°C, 2 U<sup>1</sup> of goat antibody to rabbit IgG (Calbiochem-Behring, La Jolla, CA) in 0.2 ml of incubation buffer (without normal rabbit serum) were added to each of the tubes. The tubes were incubated overnight at 4°C and centrifuged at 3,500 rpm in a Sorvall HS-4 rotor (DuPont Co., Newtown, CN) for 15 min at 10°C. The pellets were counted in a  $\gamma$ -counter; the background counts per minute from tubes without antibodies to RC proteins was determined for each of the <sup>125</sup>I-labeled antigens. The percentage of <sup>125</sup>I-labeled antigen bound by specific antibodies was calculated:

$$\% \text{ bound} = \frac{(\text{counts/min}) \text{ in pellet minus background}}{\text{total (counts/min) added minus background}}$$

The maximum percentage of bound antigen varied between 70% and 90% for five antigens. In presenting the data, the maximum for each antigen was normalized to 100%. The background for each point was <10% of the total counts for RC, LM, M, and H, and <15% for L.

### Preparation of Spheroplasts

*R. sphaeroides*, strains R-26 and 2.4.1 (ATCC 17023), were grown as previously described (3). Cells were harvested during mid to late log phase; the optical absorbance, A<sub>680</sub><sup>cm</sup>, of the medium at this stage reached a value of 0.5–1. Spheroplasts were prepared from 150 ml of liquid culture by the method of Karunairatnam et al. (30). The spheroplasts were washed with 80 ml of PBS, 20% sucrose, pH 7, and resuspended in 10 ml of PBS, 10% sucrose, pH 7.

### Labeling Spheroplasts

1 mg of affinity-purified IgG, normal rabbit IgG, or a combination of the two, totaling 1 mg in 0.5 ml of PBS, was added to 0.25 ml of the spheroplast preparation and incubated at room temperature for 1 h. Separation of the unbound IgG from the spheroplasts was achieved by applying the sample to a 38-ml 10–20% linear sucrose gradient in PBS and centrifuging at 5,000 rpm in a Spinco SW-27 rotor (Beckman Instruments, Inc.) at 4°C for 10 min and then at 10,000 rpm for an additional 10 min. The spheroplast pellet was resuspended in a 1-ml solution of ferritin (obtained from the laboratory of S. J. Singer) covalently bound to goat-anti-rabbit IgG. The ferritin conjugate was prepared by the glutaraldehyde coupling method of Kishida et al. (31) and was diluted for use in labeling studies to ~0.5 mg/ml with PBS, 20% sucrose. After incubation of the spheroplast sample with ferritin conjugate for 1 h at room temperature, the sample was layered onto a 38-ml 20–30% sucrose gradient in PBS. The unbound ferritin conjugate was separated from the spheroplasts by centrifugation at 10,000 rpm for 20 min at 4°C in a Spinco SW-27 rotor.

### Preparation of Thin Sections for Electron Microscopy

The spheroplast pellet was fixed on ice in 2% glutaraldehyde in PBS, 20% sucrose, pH 7, for 30 min. Centrifugation in a Sorvall HB-4 rotor (Beckman Instruments, Inc.) at 4°C at 4,000 rpm for 15 min using a 3-ml conical centrifuge tube produced a small pellet. Pieces of the fixed pellet were resuspended in 2% SeaPlaque (Marine Colloids Div., FMC Corp., Rockland, ME) agarose at 35°C. The agarose was solidified on ice; pieces containing the pellet were cut out, washed with cold 0.1 M potassium phosphate buffer, pH 7.4, and fixed with 2% OsO<sub>4</sub> (Ted Pella, Inc., Tustin, CA) in the same buffer on ice for 1 h. The OsO<sub>4</sub> was removed by repeated washings with cold phosphate buffer followed by distilled water. The samples were dehydrated in ethanol, the solvent was changed to propylene oxide, and the samples were embedded in Luft's mixture (32). Polymerization was carried out at 60°C overnight. Gray-to-silver sections (thickness 500–800 Å) were cut on a Reichert Om U2 ultramicrotome with a diamond knife and were picked up on 0.4% Formvar carbon-coated grids. Sections were stained for 4 min with 2% KMnO<sub>4</sub>. The grids were washed by immersing them with agitation for 10 s in a dilute solution of sodium sulfite and oxalic acid (three drops of 1.2% sodium sulfite and three drops of 1% oxalic acid by Pasteur pipet/10 ml distilled water) followed by washing with distilled water and drying. Further staining was for 4 min with lead citrate (33). The sections were examined in a Philips Model 300 transmission electron microscope at 60 kV.

<sup>1</sup> U precipitates the  $\gamma$ -globulin in 0.2 ml of 2% normal rabbit serum during a 6-h room temperature incubation (specified by manufacturer).

## RESULTS

### Determination of Antibody Specificity

The success of immunoelectron microscopy depends on the specificity of the antibodies to their respective antigens. Contamination of the antisera with antibodies directed against antigens other than the desired one can cause misleading results. It was, therefore, important to determine the level of contamination on the ferritin labeling results (discussed in the following section). There are two distinct sources for contamination of the antibodies. One arises from the presence of contaminating subunits covalently bound to the affinity column; the other is due to antibodies nonspecifically adsorbed to the affinity column.

**THE EFFECT OF CONTAMINATING SUBUNITS:** The purity of each subunit preparation was determined by SDS PAGE to >97% (see Materials and Methods). Thus, assuming equal coupling efficiency, each affinity column had at most 3% of a contaminating subunit. Sufficient amounts of antiserum were passed over the column to ensure that the dominant antigenic sites were saturated. This resulted in a contamination of the affinity-purified antibodies with at most 3% of antibodies directed against the nondominant antigen. (Note that if the dominant antigenic sites had not been saturated, a higher percentage of the eluted antibodies would be directed against the contaminating antibodies).

The purity of the final antibody preparation was tested by radioimmunoassay in which the binding of each antibody preparation to all five radiolabeled antigens (RC, LM, L, M, H) was determined. The results for anti-H that was passed over an LM-affinity column (see Fig. 1) are shown in Fig. 2. It is seen that anti-H did not bind to LM over the range of antibody concentrations (four orders of magnitude) assayed. If the binding constants of anti-LM and anti-H to their respective antigens were the same, this result shows that the fraction of antibodies directed against isolated LM subunits is  $<10^{-4}$ . We measured the binding to LM of the anti-RC population that passed through the H-affinity column (Fig. 3). The level of contamination of affinity-purified anti-H with anti-LM was determined from the ratio of concentrations of anti-LM to anti-H that bind the same amount of  $^{125}\text{I}$ -labeled LM. Comparing points A in Fig. 3 with A' in Fig. 2, this ratio is  $<10^{-4}$ . Therefore, the affinity-purified anti-H was contaminated with  $<0.01\%$  of anti-LM.

Antibodies against L and M were prepared from the same anti-LM serum<sup>2</sup> by passing it through a succession of affinity columns (see Fig. 1). To test the contamination of anti-M with anti-L and vice versa, the twice affinity-purified antibodies were again reacted with the radiolabeled contaminating subunits (Figs. 4 and 5). The level of contamination of anti-M with anti-L was determined from the ratio of concentrations of anti-L and anti-M that bind the same amount of  $^{125}\text{I}$ -labeled L. Comparing points C of Fig. 5 with C' of Fig. 4, this ratio is  $<10^{-3}$ . We conclude, therefore, that the twice affinity-purified anti-M was contaminated with  $<0.1\%$  of anti-L. A similar analysis (compare points B and B' in Figs. 4 and 5, respectively) limited the contamination of twice affinity-purified anti-L by anti-M to 0.1%.

**THE EFFECT OF NONSPECIFIC BINDING:** A potential

<sup>2</sup> This anti-LM was obtained by immunizing rabbits with LM. It is to be distinguished from the anti-LM obtained by passing anti-RC over an H-affinity column, discussed in the previous paragraph.

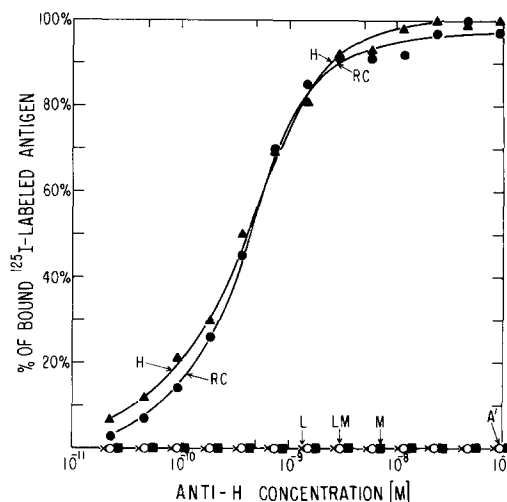


FIGURE 2 Binding of affinity-purified anti-H IgG to  $^{125}\text{I}$ -labeled antigens, RC (●), LM (○), L (×), M (■), and H (▲). Antigen concentrations  $\sim 5 \times 10^{-11}$  M. Places where three symbols (×○■) are clustered on the abscissa correspond to the same antibody concentration. These results, together with those presented in Fig. 1, show that the contamination of anti-H with antibodies against isolated LM (the dominant contamination) is  $<0.01\%$ . Immunoferritin labeling with these antibodies is shown in Fig. 6.

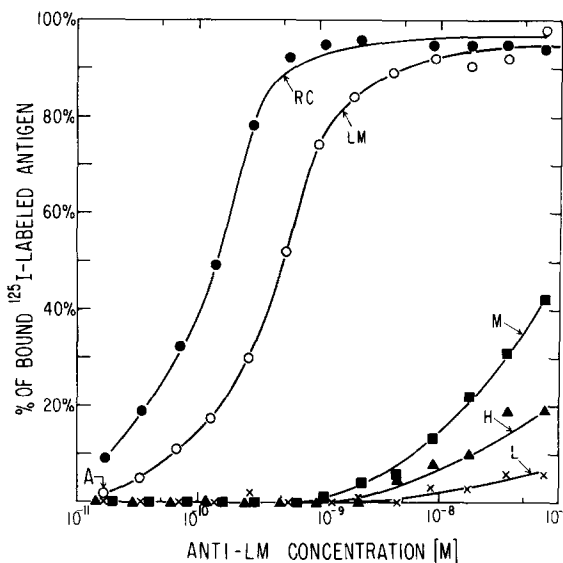


FIGURE 3 Binding of anti-LM IgG to  $^{125}\text{I}$ -labeled antigens, RC (●), LM (○), L (×), M (■), and H (▲). Antigen concentrations  $\sim 5 \times 10^{-11}$  M. Places where symbols are clustered on the abscissa correspond to the same antibody concentration. The binding of anti-LM to LM is much stronger than to L and M. This shows that during the purification of the subunits the LM complex remains essentially in its native conformation, whereas, the individual subunits are denatured and are, therefore, not well recognized by anti-LM. (Note that anti-LM refers here to anti-RC that was passed over an H affinity column, as shown in Fig. 1.)

source of contamination was antibodies nonspecifically adsorbed to the affinity columns (e.g., anti-M can bind nonspecifically to L). Since the antibodies were prepared against the native protein, some of them may not recognize the isolated, partially denatured subunits. These antibodies cannot be effectively adsorbed on affinity columns that use isolated subunits nor can their amounts be reliably estimated from radioimmu-

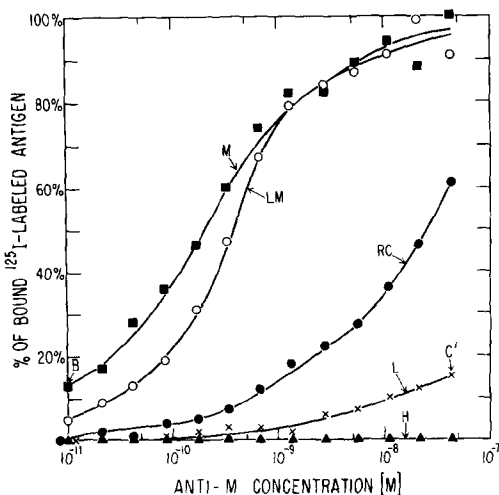


FIGURE 4 Binding of twice affinity-purified anti-M IgG to  $^{125}\text{I}$ -labeled antigens, RC (●), LM (○), L (×), M (■), and H (▲). From a comparison to points C' and C of Fig. 3, the contamination of anti-M with antibodies against isolated L was determined to be  $<0.1\%$ . Immunoferritin labeling with these antibodies is shown in Fig. 7.

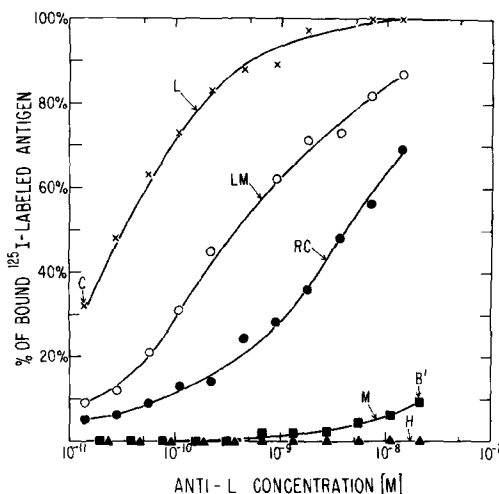


FIGURE 5 Binding of twice affinity-purified anti-L IgG to  $^{125}\text{I}$ -labeled antigens, RC (●), LM (○), L (×), M (■), and H (▲). Antigen concentrations  $\sim 5 \times 10^{-11}$  M. From a comparison of point B' with B of Fig. 4 the contamination of anti-L with antibodies against isolated M was determined to be  $<0.1\%$ . Immunoferritin labeling with these antibodies is shown in Fig. 8.

noassay. The existence of such antibodies is suggested by the data in Fig. 3 which show that anti-LM binds strongly to RC and LM but not to L and M.

The amount of nonspecific adsorption of protein was determined by passing normal rabbit serum through the columns, washing, and optically monitoring the protein eluted with acidic buffer. The nonspecific protein that bound to the first affinity column (see Fig. 1) amounted to  $<10\%$  of the affinity-purified anti-L and anti-M. The nonspecifically bound protein was characterized by SDS PAGE; the majority migrated at the same position as an IgG sample run in parallel. During the second affinity purification step (see Fig. 1),  $<1\%$  of the IgG was nonspecifically bound. This reduced the  $10\%$  contamination to  $<0.1\%$ .

In the anti-H preparation the problem of nonspecific adsorption was solved by passing the antibodies against H over an LM-affinity column. Since LM is not denatured during its

preparation, the anti-LM contamination was very effectively removed by the LM-affinity column. Thus, unlike with the anti-L and anti-M contaminants, passage through a second affinity column was not required.

### Immunoferritin Labeling

Spheroplasts of the wild type (2.4.1), as well as the mutant (R-26) bacteria, were labeled with 1 mg of each of the affinity-purified antibodies (Figs. 6, 7, and 8). Labeling experiments with reduced amounts of antibodies (plus normal rabbit IgG to keep the total amount of IgG constant) resulted in a proportional reduction in the observed labeling. Thus, the antigenic sites exposed on the membrane were not saturated with antibodies under the conditions employed in our experiments. Immunoferritin labeling of spheroplasts with 1 mg of normal rabbit IgG showed virtually no labeling. Controls that took into account the possible level of contamination were performed and are discussed below:

**ANTI-H:** Six affinity-purified anti-H preparations each from a different rabbit were used in the labeling experiments. Only one, characterized in Fig. 2, labeled both the periplasmic and cytoplasmic surface (Fig. 6). The remaining five anti-H preparations labeled the cytoplasmic membrane surface only. This result shows that the H-subunit is an asymmetrically oriented, transmembrane protein. It also illustrates the need of using many antisera when no labeling is observed (see Discussion).

The control (Fig. 6D) was an immunoferritin labeling experiment performed with 0.01 mg of anti-LM, i.e., anti-RC, from which the anti-H was adsorbed by an affinity column (characterized in Fig. 3) plus 1 mg of normal IgG. This corresponds to a contamination of the affinity-purified anti-H with  $\sim 1\%$  of anti-LM. Since this exceeds our estimated impurity level (see previous section) and no significant labeling was observed, we conclude that the immunoferritin labeling with affinity-purified anti-H was not due to contaminating antibodies.

**ANTI-M:** Antisera were obtained from ten different rabbits immunized with LM. Affinity-purified anti-M preparations were obtained from each antiserum and characterized by radioimmunoassay. Five of these singly affinity-purified anti-M preparations were used in the labeling experiments. Since all of them labeled both membrane surfaces, the remaining five antisera were not used in this set of experiments. One of the antisera was affinity-purified a second time (Fig. 1) to eliminate nonspecifically bound contaminating antibodies. It still labeled both membrane surfaces (Fig. 7). We performed immunoferritin labeling with two different controls (testing the effect of contaminating subunits and non-specific adsorption). In one, 0.01 mg of twice affinity-purified anti-L (plus 1 mg normal IgG) and, in the other, 0.01 mg of the IgG fraction of the antiserum to LM (plus 1 mg normal IgG) (Fig. 7D) were used. Neither of them showed significant labeling. Since each of these controls corresponded to more than 10 times the expected contamination (see previous section), the immunoferritin labeling with anti-M was due to antibodies specifically binding to M. We conclude, therefore, that M, like H, is a transmembrane protein.

**ANTI-L:** Affinity-purified anti-L preparations were obtained from the same antisera as the anti-M preparations (see above). All ten singly affinity-purified anti-L preparations were used to label spheroplasts. Four of these resulted in immunoferritin labeling of only the periplasmic surface of the membrane; the other six did not label significantly. One of the four prepara-

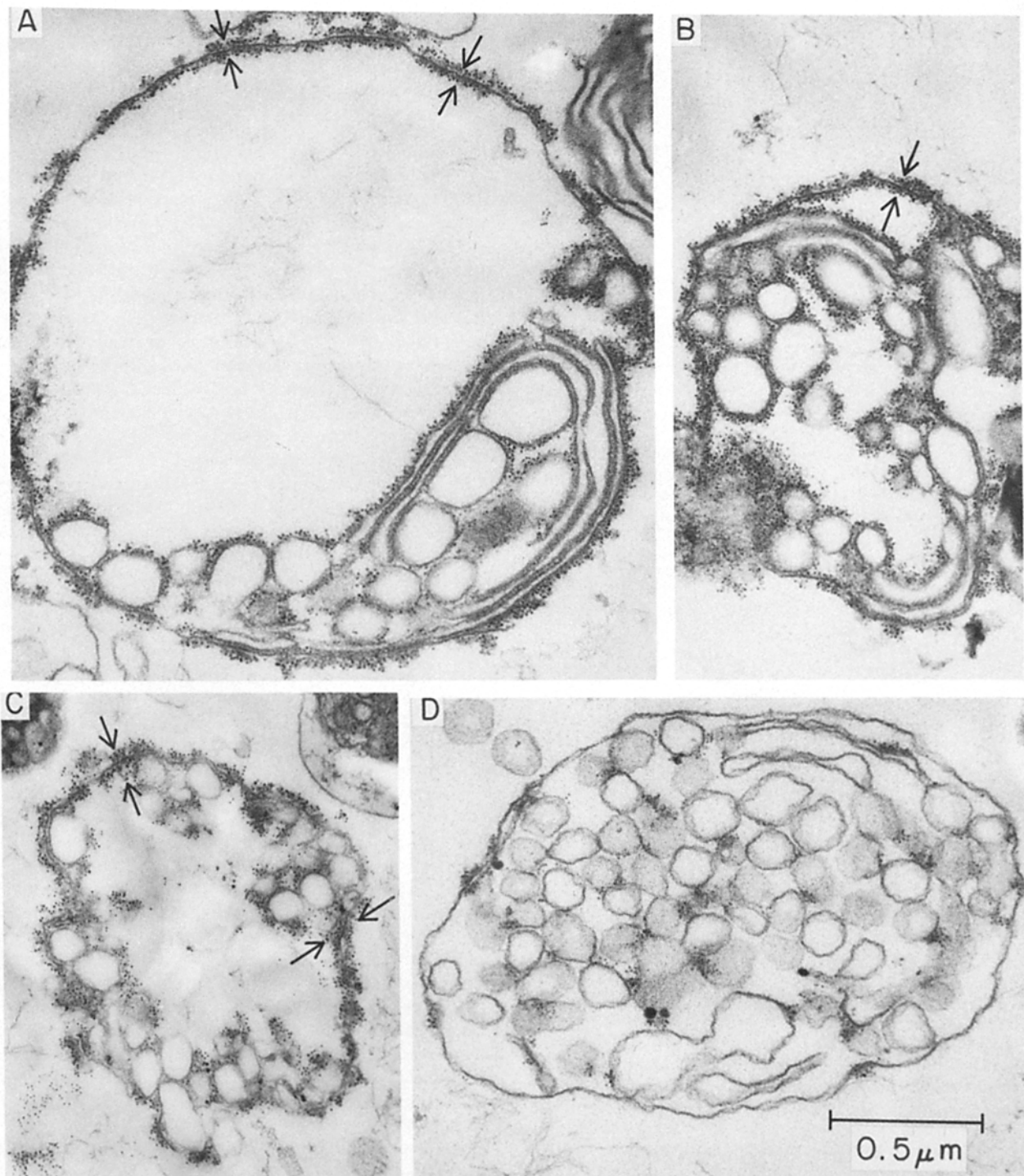


FIGURE 6 Electron micrographs of *R. sphaeroides* spheroplasts incubated with affinity-purified rabbit anti-H antibodies (1 mg) (characterized in Fig. 2) and immunoferritin labeled with goat anti-rabbit ferritin conjugate. Arrows correspond to regions where the membrane surface is clearly labeled. (A and B) *R. sphaeroides*, strain R-26. (C) *R. sphaeroides*, strain 2.4.1. (D) Control: *R. sphaeroides*, strain R-26, incubated with 1 mg of normal rabbit IgG and 0.01 mg of anti-RC IgG passed over an H-affinity column. The concentration of anti-LM in this mixture exceeds, at least by an order of magnitude, the estimated level of contamination of anti-H used in A, B, and C. Thus, H is clearly a transmembrane protein.  $\times 50,000$ .

tions that labeled was affinity-purified a second time. It still labeled the periplasmic membrane surface (Fig. 8). Immunoferritin labeling with either 0.01 mg of twice affinity-purified anti-M (specific contamination control) or 0.01 mg of anti-LM IgG (control for nonspecific binding, Fig. 8 D) gave no significant labeling. Since each of these controls corresponded again to more than 10 times the maximum expected contamination,

the immunoferritin labeling with anti-L was due to antibodies specifically binding to L. We conclude that L is exposed at least at the periplasmic membrane surface.

#### DISCUSSION

We used antibodies specific to the three subunits of RCs from *R. sphaeroides* to determine the topography of the subunits in



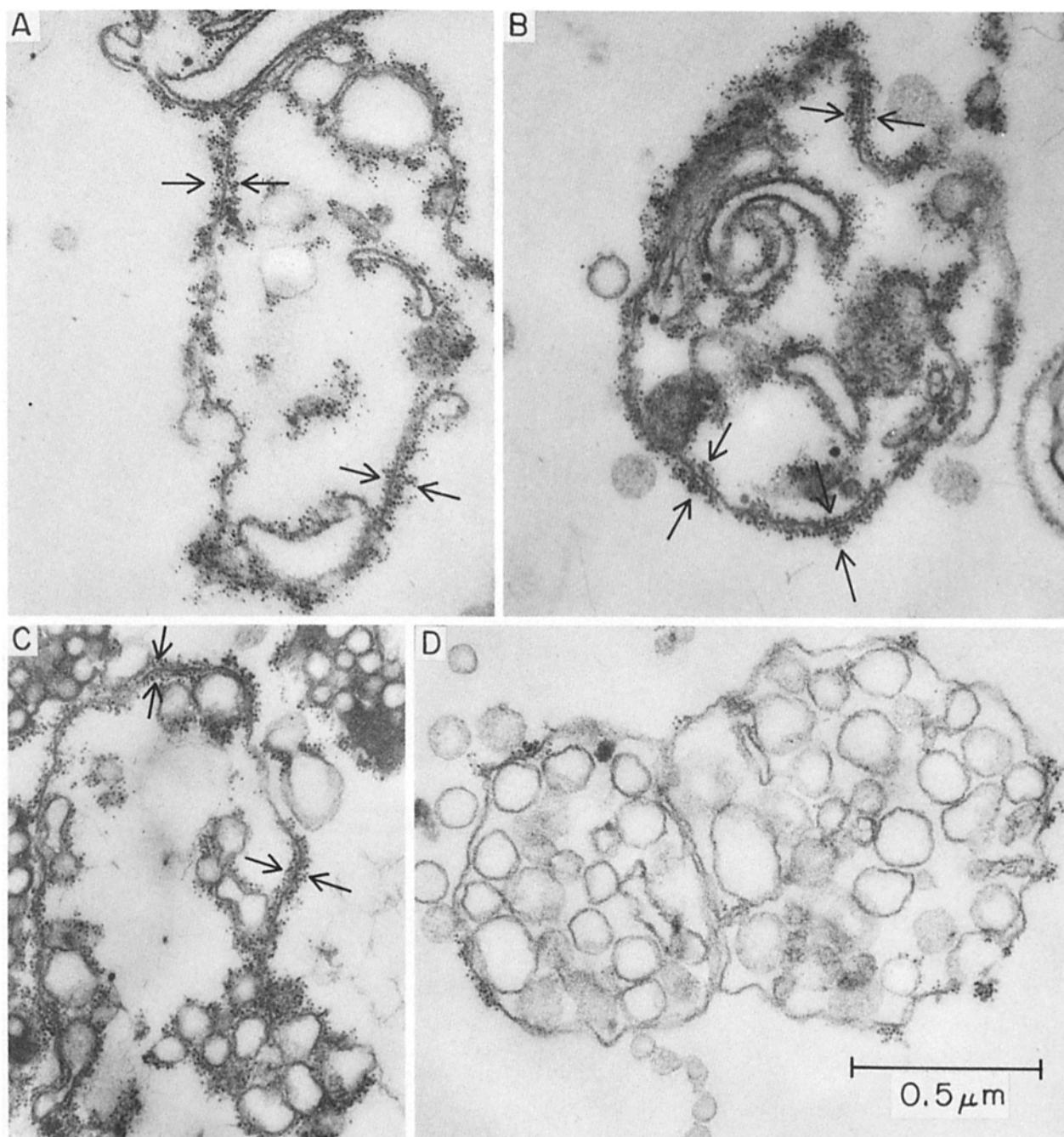


FIGURE 7 Electron micrographs of *R. sphaeroides* spheroplasts incubated with twice affinity-purified anti-M antibodies (characterized in Fig. 4) and immunoferritin labeled with goat anti-rabbit ferritin conjugate. Arrows correspond to regions where the membrane surface is clearly labeled. (A and B) *R. sphaeroides*, strain R-26. (C) *R. sphaeroides*, strain 2.4.1. (D) Control: *R. sphaeroides*, strain R-26, incubated with 1 mg normal rabbit IgG and 0.01 mg of the IgG fraction of the antiserum to LM. A control using 0.01 mg of twice affinity-purified anti-L (plus 1 mg of normal IgG) also produced negligible labeling (not shown). Both of these controls had about 10 times the estimated contamination of anti-M used in A, B, and C. Thus, the labeling in A, B, and C is due to M.  $\times 60,000$ .

the membrane of spheroplasts. There are two requirements for a successful labeling experiment with antibodies: a high degree of specificity of the antibodies to a particular subunit; and strong binding to the subunits in the native protein to withstand the manipulations of the spheroplasts after addition of antibodies. Immunoabsorption techniques were used to purify the subunits and antibodies (affinity chromatography) as well as to assay the antibodies (radioimmunoassay).

Antibodies produced by injecting rabbits with isolated sub-

units bound only weakly to RCs. These antibodies, therefore, did not satisfy the second requirement. This situation is commonly encountered whenever protein subunits are denatured during their purification. Consequently, when used as immunogens the resulting antibodies bind strongly to the denatured antigen but recognize only weakly the native form. This difficulty was overcome by producing antisera against native RCs and LM and exploiting the fact that a subpopulation of antibodies in these antisera have a sufficiently great affinity for the

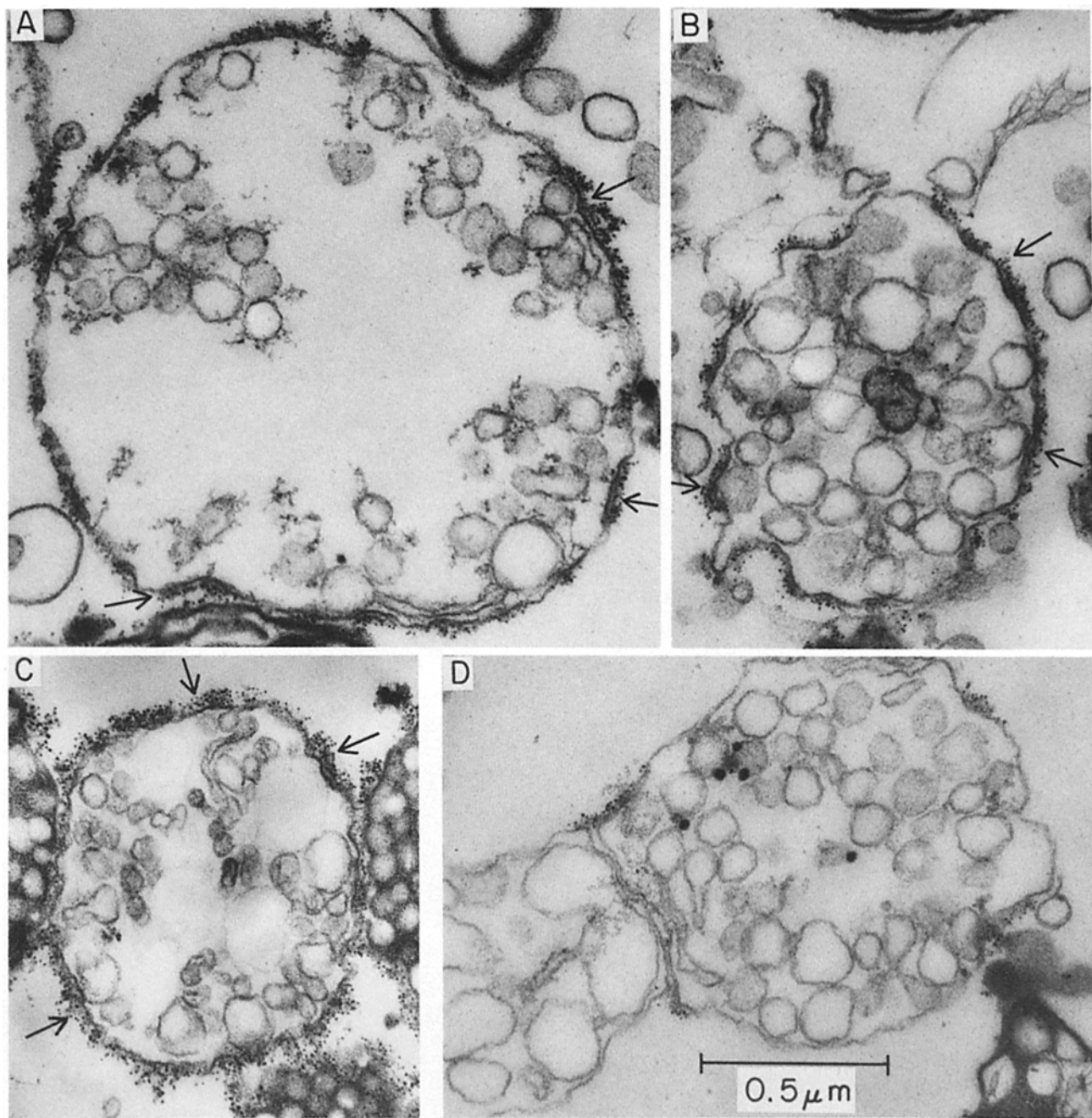


FIGURE 8 Electron micrographs of *R. sphaeroides* spheroplasts incubated with twice affinity-purified anti-L antibodies (characterized in Fig. 5) and immunoferritin labeled with goat anti-rabbit ferritin conjugate. Arrows correspond to regions where the membrane surface is clearly labeled. (A and B) *R. sphaeroides*, strain R-26. (C) *R. sphaeroides*, strain 2.4.1. (incubated with singly affinity-purified anti-L). (D) Same as described in Fig. 7; a second control using 0.01 mg twice affinity-purified anti-M also gave no significant labeling (not shown). Note that only the periplasmic surface of the membrane was found to be labeled.  $\times 60,000$ .

isolated subunits to bind to immunoadsorption column containing L or M. The antibodies prepared by this procedure bound more strongly to the subunits in the protein than to the isolated ones. The success of this strategy is based on the fact that the requirement for the strength of binding in the affinity purification procedure is less severe than in the ferritin labeling, which involves several washings and centrifugations.

Before discussing the detailed topography of the RC we wish to comment on a gross feature of the labeling results. We found that labeling occurred along almost the entire cytoplasmic (CM) membrane (see, for example, Fig. 6A). Unlabeled patches on the outside of the CM are probably due to incomplete

removal of cell walls in the preparation of the spheroplasts; unlabeled patches on the inside of the CM presumably arise from steric hindrances of other structures that pressed against the membrane during the time of ferritin labeling. (Subsequent treatment of spheroplasts swells them and changes their morphology.) The lack of labeling on the inside of the chromatophore structures (see Figs. 6-8) is believed to be due to the narrow neck of the invaginations that prevent entry of the antibodies. In many instances, we have seen complete labeling of the entire CM (not shown). These findings show that RCs are distributed throughout the CM. This result is in contradiction with the conclusions reached by Parks and Niederman



(34) who reported that the RCs are confined to the intracytoplasmic membrane (ICM) (from which chromatophores are derived). Since the labeling experiments are not quantitative, a concentration gradient of the RCs between the CM and ICM cannot be excluded and could account for the seemingly contradictory results. Several other differences in the chemical composition of the CM and ICM have been reported (for a recent review, see, for example, reference 35).

We now turn to the topography of the RC and its subunits. Figs. 6, 7, and 8 show the results of the ferritin labeling experiments. Antibodies to H and M labeled both surfaces of the membrane (Figs. 6 and 7) demonstrating that both these subunits are transmembrane proteins. Antibodies to L labeled only the periplasmic surface of the membrane (Fig. 8). However, absence of labeling is not meaningful since it may only demonstrate that no probes (e.g., antibodies) recognizing a particular protein segment are present or that the site is not accessible to the probe. Thus, a positive labeling result is necessary (and sufficient) to draw a definitive conclusion concerning the topography.

Only one of six antibody preparations against H labeled the periplasmic surface of the membrane; all of them labeled the cytoplasmic surface. This result shows that H is asymmetrically oriented in the membrane. It precludes an alternate explanation that H is merely distributed symmetrically on both sides of the membrane without crossing (spanning) it.

The strength of labeling decreased from H to M to L. This is in accord with the polarity of the subunits (36), H being the most polar. This result is not surprising since one would expect the more polar subunits to have a larger portion exposed to the aqueous phase outside the membrane. A quantitative measure of the degree of polarity is given by the "hydropathy indices" (37) which are  $-0.08$ ,  $+0.32$ , and  $+0.51$  for H, M, and L, respectively (the higher the number, the lower the polarity).

The weak labeling of L and, to some extent, the M subunit is probably responsible for the failure of most workers to localize these subunits unambiguously (15–20), whereas H has been shown consistently to be exposed on the cytoplasmic surface of the membrane. Francis and Richards (17) reported weak labeling of L on the cytoplasmic surface of the membrane but were unable to confirm the labeling of any of the subunits on the periplasmic surface. Since their results differ from ours, we shall comment in more detail on their experiments. The method that they used involved radioactive labeling (with pyridoxal phosphate plus [ $^3\text{H}$ ]KBH<sub>4</sub>) of both "inside-out" chromatophores and "right-side-out" spheroplast-derived vesicles. After labeling both types of vesicles, the proteins were extracted and subjected to SDS PAGE. The radioactivity of the bands was assayed by radioautography. They observed radioactivity at the position of the M-subunit in the electrophoretograms of radiolabeled spheroplast-derived vesicles. However, purified RCs derived from the same vesicles did not show radioactivity at the position of the M-subunit. The authors, therefore, suggested that the radioactivity observed when the vesicles were used was due to proteins that were not associated with the RC but had the same molecular weight as the M-subunit. This illustrates the hazard of identifying a protein solely by its migration in SDS PAGE. Thus, the weak radioactivity in the "L-band" when labeled chromatophores were subjected to SDS PAGE could similarly have been due to a protein that was not associated with the RC.

An extensive analysis of the topography of RCs of *R. sphaeroides* was recently published by Bachmann et al. (18). These

authors investigated chromatophores and spheroplast-derived vesicles by proteolysis and radioiodination. They reported labeling of all three subunits on both sides of the membrane, although they point out that the extent of surface exposure of L is less certain than that of M and H. Their SDS PAGE work suffers from the same uncertainty as that of Francis and Richards (17), discussed above. Their main conclusion, however, that the RC is an asymmetric transmembrane protein is in agreement with our results.

The transmembrane nature of the RC is consistent with its function in the photosynthetic membrane. The hole and electron generated by light during the primary charge separation in the RC are vectorially channeled to a secondary electron donor (cytochrome  $c_2$  [cyt  $c_2$ ]) located on the periplasmic side of the membrane (38) and to a secondary acceptor (ubiquinone) that has been implicated in the uptake of protons at the cytoplasmic side of the membrane (39–41). The oriented transmembrane nature of the electron transport is in accord with Mitchell's chemiosmotic theory (8) and suggests that the driving element of cyclic electron transport, the RC, should be an oriented transmembrane protein. We have shown that this is indeed the case.

Recent functional assays of electron transport have provided independent and corroborative evidence concerning the topography of the M and L subunits. Chemical cross-linking of cyt  $c_2$  with purified RCs localized the cyt  $c_2$ -binding site close (to within  $\sim 10$  Å) to the L and M subunit (42). These results, together with the known presence of cyt  $c_2$  in the periplasmic space (36), suggest that L and M protrude from the membrane on the periplasmic side where they are contacted by cyt  $c_2$ . On the acceptor side, both the primary (43) and secondary (44) ubiquinone were localized at or near the M subunit. Since the protein uptake associated with the secondary quinone occurs on the cytoplasmic side of the membrane (39–41), M must be exposed on that side as well. These considerations lead to the conclusion that M is a transmembrane protein, as indeed was found in the ferritin labeling experiments.

A logical extension of this work would be to obtain the sequence of the polypeptide segments that protrude from the membrane. In particular, since the N-terminal sequence of all three subunits has been determined (45), their localization, with respect to the bacterial membrane, could be obtained by exposing vesicles with either sidedness, i.e., chromatophores and spheroplast-derived vesicles, to proteolytic enzymes and analyzing the sequence of the cleaved products.

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