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Antigenic Architecture of Membrane Vesicles from *Escherichia coli*†

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With the Technical Assistance of Helen Doherty

ABSTRACT: The antigenic architecture of membrane vesicles prepared from *Escherichia coli* ML 308-225 has been studied using crossed immunoelectrophoresis. Progressive immunoadsorption experiments conducted with control vesicles and with physically disrupted vesicles were used to monitor and quantitate the expression of 14 different immunogens. Eleven immunogens, including NADH dehydrogenase (EC 1.6.99.3), D-lactate dehydrogenase (EC 1.1.1.27), dihydro-orotate dehydrogenase (EC 1.3.3.1), 6-phosphogluconate dehydrogenase (EC 1.1.1.43), polynucleotide phosphorylase (EC 2.3.7.8), and β-galactosidase (EC 3.2.1.23), exhibit minimal expression (10% or less) unless the vesicles are disrupted. Three unidentified antigens are expressed to a similar extent in untreated and disrupted vesicles. Consideration of these and other results [Owen, P., & Kaback, H. R. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3148] in terms of membrane polarity, dislocation of antigens, and possible transmembrane orientation of some immunogens reveals that over 95% of the membrane in the vesicle preparations is in the form of sealed sacculi with the same orientation as the intact cell. Furthermore, antigens are distributed across the membrane in a highly asymmetric manner, indicating that dislocation of components from the inner to the outer surface of the membrane during vesicle preparation does not occur to an extent exceeding 10%.

In previous communications (Owen & Kaback, 1978, 1979), a complex crossed immunoelectrophoresis (CIE)[1] reference pattern was established for membrane vesicles prepared from *Escherichia coli* ML 308-225, and many of the 52 component immunogens were characterized. In addition, results of progressive immunoadsorption experiments with certain vesicle immunogens were presented and the relationship

$$\frac{1}{A_v} = \frac{1}{A_0} - Kxv \qquad (1)$$

was derived and shown to apply to the data (Owen & Kaback, 1978) where A_0 and A_v are the peak areas subtended by an immunoprecipitate i following adsorption with 0 and v mL of a vesicle suspension, respectively. K is a constant under the experimental conditions adopted, and x is the degree to which antigen i is expressed in the membrane suspension used for adsorption. Comparison of the values of x for antigen i following adsorption with intact and disrupted vesicles reveals the extent to which i becomes expressed following disruption or, stated conversely, the extent to which i is not expressed in intact vesicles. By using this approach quantitatively, the distribution of i on the inner and outer surface of the membrane can be deduced (Owen & Kaback, 1978).

In this paper, the results of an extensive series of quantitative immunoadsorption experiments of the type outlined above are presented, and the distribution of 14 antigens across the vesicle membrane is established. It is apparent from these and other data (Owen & Kaback, 1978) that the vesicle membrane of *E. coli* ML 308-225 is markedly asymmetric with respect to its antigenic architecture and that it retains the topology of the parent cell. Moreover, dislocation of membrane components from the inner to the outer surface of the membrane occurs to a minimal extent for all of the antigens tested.

Experimental Procedures

Methods

Growth of Cells and Preparation of Membrane Vesicles. *E. coli* ML 308-225 ($i^-z^-y^+a^+$) was grown on minimal medium with 1.0% disodium succinate (hexahydrate) and membrane vesicles were prepared as described (Kaback, 1971; Short et al., 1975). Vesicles were suspended in 0.1 M potassium phosphate (pH 6.6), frozen in liquid nitrogen, and maintained at or below −70 °C. Prior to use in adsorption experiments, suspensions were thawed rapidly at 45 °C. When appropriate, the vesicles were washed by centrifugation at 30000*g* for 30 min at 4 °C followed by careful resuspension to their original volume in 0.1 M potassium phosphate (pH 6.6). Concentration was achieved by centrifugation in a similar manner and resuspension of the vesicles in a reduced volume of 0.1 M potassium phosphate, pH 6.6.

Disruption of Membrane Vesicles. Sonic disruption of membrane vesicles was performed at 0 °C using 30-s or 1-min

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[1] Abbreviations used: CIE, crossed immunoelectrophoresis; $NaDodSO_4$, sodium dodecyl sulfate.

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pulses from an MSE 100-W ultrasonic disintegrator fitted with a $^3/_8$-in. probe (Salton & Netschey, 1965). Disruption was also achieved by three passages through an Aminco French pressure cell at 6500 psi. Dispersal of membranes in detergent was accomplished by addition of either Triton X-100 or sodium dodecyl sulfate ($NaDodSO_4$) to final concentrations of 0.5% (v/v) or 0.3% (w/v), respectively.

Immunological Procedures. Methods for preparing concentrated anti-vesicle immunoglobulins and Triton X-100–EDTA extracts of membrane vesicles and for performing CIE have been given (Owen & Kaback, 1978, 1979). Peak areas subtended by individual immunoprecipitates were estimated by either (a) photocopying stained immunoplates and subsequently excising and weighing ten copies of each peak (photocopy paper routinely weighed 7.89 mg/cm^2) or (b) using a compensating polar planimeter (Owen & Kaback, 1979).

Adsorption of Anti-Vesicle Immunoglobulins. Prior to adsorption, concentrated immunoglobulins, which were routinely stored at 4 °C in 0.1 M NaCl containing 15 mM NaN_3, were extensively dialyzed against 0.1 M potassium phosphate (pH 6.6). In experiments designed to assess the effect of washing on vesicle integrity, concentrated anti-vesicle immunoglobulins (0.54 mL, 85 mg of protein) were incubated with frequent shaking for 1 h at 20 °C with 2.0 mL of control vesicles or with 2.0 mL of vesicles that were washed a given number of times. Vesicles were removed by centrifugation (30000*g* for 30 min at 4 °C) and the supernatant fractions containing unadsorbed immunoglobulins were dialyzed against 0.1 M NaCl containing 15 mM NaN_3. In adsorption experiments designed to assess the molecular architecture of the vesicle membrane, 0.5-mL aliquots of the immunoglobulin preparation were incubated in a similar manner with 0–1.5-mL samples of untreated or disrupted vesicles (containing 0–18 mg of protein) and sufficient buffer (0.1 M potassium phosphate, pH 6.6) to give a final volume of 2.0 mL. Unadsorbed immunoglobulins were then processed as described above.

Protein Determinations. Protein determinations were carried out as described (Dulley & Grieve, 1975) with bovine serum albumin as standard.

Materials

Agarose was obtained from Miles Laboratories and Triton X-100 from Research Products International.

Results

Expression of Antigens in Intact and Disrupted Membrane Vesicles. Results from a series of progressive adsorption experiments conducted with untreated and disrupted ML 308-225 vesicles are presented graphically in Figures 1 and 2 where the reciprocal of the peak area subtended by ten discrete immunoprecipitates (i.e., $1/A$) is plotted as a function of the quantity of vesicles used during immunoadsorption (expressed as volume, v). Clearly, as predicted by eq 1, there is a linear relationship between $1/A$ and v for most of the antigens under the adsorption conditions used. Only when values of $1/A$ fall below about $^1/_2A$ is deviation from linearity observed in some instances (cf. Figure 2). The value of $^1/_2A$ corresponds to a point in the adsorption experiments where about 50% of the relevant antibody is removed (Weeke, 1973; Owen & Kaback, 1978). A primary assumption in the derivation of eq 1 is that the amount of antibody adsorbed is proportional to the amount of adsorbing antigen available (Owen & Kaback, 1978). While it is clear from a consideration of typical quantitative precipitin tests (Heidelberger & Kendall, 1935) that this assumption is valid under conditions of moderate antibody excess, the relationship may not hold as equivalence is approached (i.e., as $1/A$ tends to zero). The unique immunoprecipitin profile exhibited by antigen no. 28 following adsorption with $NaDodSO_4$-treated vesicles (Figure 1D) will be discussed.

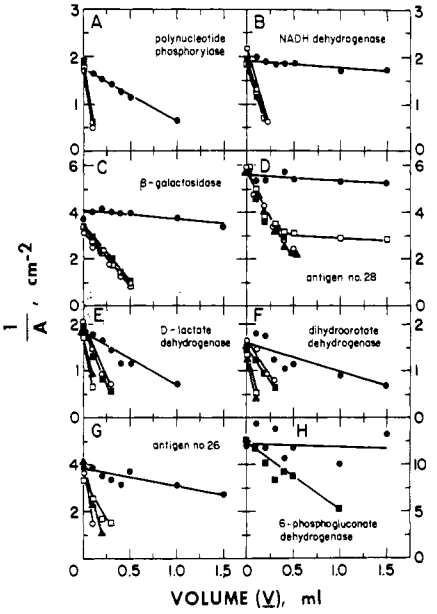

FIGURE 1: Effect of progressive adsorption of anti-vesicle immunoglobulins with control and disrupted vesicles on the peak areas of immunoprecipitates corresponding to the following antigens: polynucleotide phosphorylase (antigen no. 5; panel A), NADH dehydrogenase (antigen no. 19/27; panel B), β-galactosidase (antigen no. 8; panel C), antigen no. 28 (panel D), D-lactate dehydrogenase (antigen no. 45; panel E), dihydro-orotate dehydrogenase (antigen no. 41, panel F), antigen no. 26 (panel G), and 6-phosphogluconate dehydrogenase (antigen no. 7; panel H). Anti-vesicle immunoglobulin (89 mg of protein) was adsorbed with increasing volumes (v) of an untreated membrane vesicle suspension (12 mg of protein per mL) as described in Methods (●). Similar experiments were conducted with identical aliquots of vesicles that had been disrupted by sonication for 5 min (○), passage through a French pressure cell (■), dispersion in $NaDodSO_4$ (□) or dispersion in Triton X-100 (▲). The adsorbed immunoglobulin was incorporated into agarose gels (75 μL per mL) and analyzed against a Triton X-100–EDTA extract of membrane vesicles (28 or 5.6 μg of protein) by CIE. The peak areas (A) were estimated from immunoplates that were stained either with Coomassie brilliant blue or by zymogram techniques (Owen & Kaback, 1979). Peak areas obtained from gels run at the lower antigen concentration (specifically those relating to D-lactate dehydrogenase and dihydro-orotate dehydrogenase antigens) were normalized to correspond to an antigen concentration of 28 μg of membrane protein.

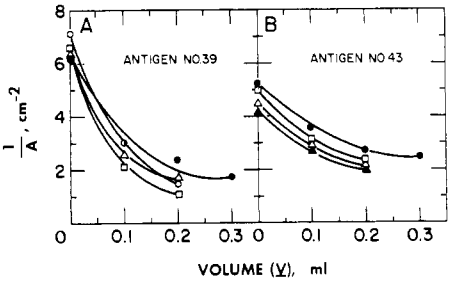

FIGURE 2: Effect of progressive adsorption of anti-vesicle immunoglobulins with control and disrupted vesicles on the peak areas of immunoprecipitates corresponding to antigen no. 39 (panel A) and antigen no. 43 (panel B). Adsorption was conducted as in Figure 1 using untreated veiscles (●), or vesicles that had been disrupted by sonication for 30 s (▲) or 5 min (○), or solubilization in $NaDodSO_4$ (□), or Triton X-100 (▲). Other experimental details are given in Figure 1. Practical difficulties were encountered in accurately estimating peak areas obtained following CIE analysis of immunoglobulin adsorbed with more than 0.3 mL of membrane vesicles.

Table I: Effect of Vesicle Disruption on Expression of Immunogens

immunogen	ratio[a]			
	x_{FP}/x_{con}	$x_{son.}/x_{con}$	$x_{NaDodSO_4}/x_{con}$	x_{TX}/x_{con}
polynucleotide phosphorylase	12.5	11.0	10.4	ND[b]
NADH dehydrogenase (antigen no. 19/27)	45.4	54.3	45.9	ND
β-galactosidase	19.3	18.3	20.5	ND
D-lactate dehydrogenase	5.6	5.4	10.8	9.2
dihydro-orotate dehydrogenase	5.2	5.3	14.9	15.9
6-phosphogluconate dehydrogenase	22.0	ND	ND	ND
antigen no. 28	45.2	44.6	45[c]	45.4
antigen no. 26	ND	32.3	11.8	22.1
antigen no. 39	ND	1.20[c]	1.30[c]	1.26[c]
antigen no. 43	ND	0.95[c,d]	1.05[c]	0.85[c]

[a] x_{con}, x_{FP}, $x_{son.}$, $x_{NaDodSO_4}$, and x_{TX} represent the extents to which the antigen in question is expressed in control, French pressed, sonicated (5 min), $NaDodSO_4$-treated, and Triton X-100 treated membrane vesicles, respectively. Data were computed from the slopes of the $1/A$ vs. v functions shown in Figures 1 and 2 and from other similar experiments. [b] ND, not determined. [c] Values calculated from the initial slope of $1/A$ vs. v. [d] Value relates to vesicles that were sonicated for 30 s.

One clear and important feature of the immunoadsorption data presented is that the vesicle immunogens fall into two distinct categories: (i) those shown in Figure 1 [polynucleotide phosphorylase (antigen no. 5; panel A), NADH dehydrogenase (antigen no. 19/27; panel B), β-galactosidase (antigen no. 8, panel C), antigen no. 28 (panel D), D-lactate dehydrogenase (antigen no. 45; panel E), dihydro-orotate dehydrogenase (antigen no. 41; panel F), antigen no. 26 (panel G), and 6-phosphogluconate dehydrogenase (antigen no. 7; panel H)]; and (ii) those shown in Figure 2 [antigen no. 39 (panel A) and antigen no. 43 (panel B)]. Categorization into these two groups is clearly justified by comparing the slopes of $1/A$ vs. v for each immunogen in intact and disrupted vesicles. The antigens shown in Figure 1 exhibit dramatically increased slopes following adsorption with disrupted vesicles. In contrast, the antigens shown in Figure 2 exhibit similar slopes after adsorption with either intact or disrupted vesicles. In other words, the membrane immunogens in the first group adsorb antibody relatively ineffectively unless the vesicles are disrupted, while those in the second group adsorb antibody equally effectively whether the vesicles are intact or disrupted.

As discussed previously (Owen & Kaback, 1978) and outlined above, an accurate, quantitative assessment of the degree to which disruption increases the availability of an antigen to antibodies can be obtained by calculating the ratio of $x_{disrupted}$ to $x_{control}$ (i.e., the ratio of the slopes of $1/A$ vs. v for disrupted and control vesicles). Values computed in this fashion for ten vesicle immunogens are given in Table I. Several points are noteworthy. With few exceptions, the four disruption techniques utilized (i.e., sonication for 5 min, French pressing, treatment with $NaDodSO_4$, and treatment with Triton X-100) affect the expression of any given antigen to a similar extent. D-Lactate dehydrogenase and dihydro-orotate dehydrogenase, however, appear to be expressed two to three times more effectively following detergent treatment relative to disruption by physical techniques (Figure 1, panels E and F, respectively). This observation might be taken to imply that these two antigens are unique in that treatment of vesicles with detergents exposes additional determinants that are not accessible to antibodies following exhaustive sonication or repeated passage through a French pressure cell. On the other hand, since both enzymes are weakly immunogenic, it is more likely that antibodies directed against these components may face severe competition from other antibodies for binding in situ unless steric constraints are fully relieved by detergent solubilization.

Another notable feature of the data presented in Table I is that disruption of the vesicles affects expression of certain antigens to different extents. Clearly, antigens no. 39 and 43 are expressed to a similar degree in intact and disrupted vesicles; however, the degree to which other antigens become expressed following vesicle disruption varies from about 10-fold for D-lactate dehydrogenase (the value pertains to detergent solubilized vesicles) to about 50-fold for NADH dehydrogenase (antigen no. 19/27). Thus, the antigens in the latter group are accessible to relatively small but differing extents in untreated membrane vesicles. Values computed from Table I indicate that the percentage of antigen molecules accessible to antibody in intact vesicles is as follows: polynucleotide phosphorylase, about 9%; NADH dehydrogenase (antigen no. 19/27), about 2%; β-galactosidase, about 5%; D-lactate dehydrogenase, about 10%; dihydro-orotate dehydrogenase, about 6%; 6-phosphogluconate dehydrogenase, about 5%; antigen no. 28, about 2%; and antigen no. 26, about 5%. In contrast, the corresponding values for antigens no. 39 and 43 are 82% and 105%, respectively. Similar data on the expression of four additional immunogens have been presented elsewhere (Owen & Kaback, 1978), and about 11% and 2% respectively of the membrane ATPase (EC 3.6.1.3; antigen no. 12) and NADH dehydrogenase (antigen no. 15) and essentially 100% of Braun's lipoprotein (antigen no. 49) and antigen no. 47 are accessible to antibodies in intact vesicles. Quantitative information on the expression of other antigens in the reference pattern is difficult to obtain due to the faint nature of many immunoprecipitates, the complexity of certain regions of the immunoprecipitin pattern, and the extremes in peak area exhibited by some of the immunoprecipitates. In any event, it is apparent from a visual comparison of relevant immunoplates that antigens no. 18, 21, and 37 are expressed to a minimal extent on the surface of intact vesicles, while antigen no. 22 is expressed to the same extent in control and disrupted vesicles.

Effect of Centrifugation and Resuspension on Vesicle Integrity. During preparation, membrane vesicles are subjected to frequent cycles of centrifugation and resuspension, operations that conceivably could alter their integrity. For this reason, adsorption experiments were performed with control vesicle preparations and with preparations that were centrifuged and resuspended in buffer from one to four times. If vesicle structure is altered by these procedures, alterations in the peak areas of the immunoprecipitates would be expected following CIE analysis of the adsorbed immunoglobulins. Since this phenomenon was not observed (data not shown), it may be concluded that centrifugation and resuspension do not alter vesicle structure with respect to the topological distribution of immunogens.

Discussion

In a preliminary communication (Owen & Kaback, 1978), the distribution of four immunogens [ATPase, NADH dehydrogenase (antigen no. 15), Braun's lipoprotein, and antigen no. 47] across the membrane of *E. coli* M 308-225 vesicles was reported, and it was concluded that the majority of the vesicles are in the form of sealed sacculi with basically the same configuration as the membrane in the intact cell. In this paper, the topological distribution of 14 additional membrane immunogens is established. The data, taken as a whole, lead to the following generalizations: (i) membrane immunogens in ML 308-225 vesicles fall into two distinct categories which will be called groups 1 and 2 for convenience; (ii) group 1 immunogens [polynucleotide phosphorylase, two or three NADH dehydrogenases (antigens no. 15 and 19/27), β-galactosidase, D-lactate dehydrogenase, dihydro-orotate dehydrogenase, 6-phosphogluconate dehydrogenase, ATPase, and antigens no. 18, 21, 26, 28, and 37] are expressed to a minimal extent on the vesicle surface unless the membranes are disrupted; (iii) group 2 immunogens (Braun's lipoprotein and antigens no. 22, 39, 43, and 47) are expressed to a similar extent in intact and disrupted vesicles; and (iv) in intact vesicles, expression of group 1 immunogens varies from about 2% for the immunoprecipitates exhibiting NADH dehydrogenase activity to about 10–11% for D-lactate dehydrogenase and ATPase.

Antigens in group 1 include enzymes associated with the inner surface of the plasma membrane in intact cells (e.g., D-lactate dehydrogenase, NADH dehydrogenase, and ATPase) and also cytoplasmic enzymes (e.g., β-galactosidase). Furthermore, group 2 antigens include a component known to be located exclusively in the outer regions of the cell envelope (i.e., Braun's lipoprotein; Bosch & Braun, 1973). Thus, it seems reasonable to conclude on the basis of these data alone that the vesicle membrane is markedly asymmetric with regard to its antigen architecture and that the vesicles themselves are largely intact and retain the topology of the intact cell.

There are at least two possible explanations for the variation observed in the expression of group 1 antigens in untreated vesicle preparations. It is possible that about 90% of the membrane is in the form of correctly oriented vesicles and that the remaining 10% is heterogeneous in the sense that it consists of inside-out vesicles and/or open sheets of membrane that have lost a significant proportion of some of their membrane components. While it is impossible to rule out this interpretation on the basis of the present data, this possibility is not entirely compatible with previous results suggesting the absence of inside-out vesicles (cf. Kaback et al., 1977). Moreover, it is difficult to explain why some membrane-associated enzymes (e.g., antigen no. 15) would be lost almost entirely from a small percentage of the total membrane, while other membrane-associated enzymes (e.g., ATPase) are not. A more likely explanation is that about 98% of the membrane in the vesicle suspension is in the form of correctly oriented, sealed sacculi that are impermeable to antibody, and that various enzymes and antigens become dislocated to small but differing extents during vesicle formation. Certainly, this interpretation is compatible with earlier data regarding the structure of membrane vesicles from *E. coli* ML 308-225 (Short et al., 1975) and also with data from other laboratories indicating that dislocation can occur (Weiner, 1974; Futai, 1974; Futai & Tanaka, 1975; Wickner, 1976; Adler & Rosen, 1977; Yamato et al., 1978). It should be stressed, however, that we have been unable to detect an antigen that is dislocated to an extent exceeding about 11%.

It could be argued that the expression of some group 1 immunogens on the surface of the intact vesicle might reflect a transmembrane orientation rather than dislocation. However, this reasoning seems untenable for the following reasons. Clearly, some of the antigens in question (e.g., β-galactosidase and antigen no. 26) are of cytoplasmic origin (Owen & Kaback, 1979). Furthermore, since expression on the vesicle surface does not exceed 11% for any group 1 antigen tested, it follows that most of the major antigenic determinants on prospective transmembrane proteins must be located on the cytoplasmic face of the membrane. Thus, progressive adsorption of anti-vesicle serum with untreated vesicles would result in initial, rapid removal of antibodies directed against surface determinants, but no removal of immunoglobulins directed toward determinants on the same molecule that are expressed on the inner face of the vesicle membrane. The $1/A$ vs. v plots would be distinctly biphasic, exhibiting a rapid initial decrease followed by a second phase with a negligible slope. This phenomenon has not been observed. It should be emphasized, however, that the data do not rule out the possibility of a transmembrane component with weakly antigenic sites on the outer surface of the membrane or a transmembrane component with qualitatively similar determinants on both sides of the membrane.

While it is clear that dislocation probably accounts in large part for the expression of group 1 immunogens in control vesicles, it is less apparent why the extent of dislocation varies with different antigens. Although the mechanism of dislocation is not understood, it might involve readsorption of a soluble form of the component in question to the outer surface of the membrane during spheroplast rupture. If so, dislocation would depend, among other things, on the equilibrium between free and bound forms of the antigen in the cell lysate and also on the affinity of the antigen for the outer surface of the membrane. In any case, it is clear from results of adsorption experiments conducted with washed vesicles that the dislocation event is probably restricted to spheroplast rupture, since repeated centrifugation and resuspension induces neither further topological redistribution of membrane antigens nor significant alteration in the permeability of the vesicles to antibody.

All the group 2 immunogens are probably expressed on the outer surface of the membrane vesicles. Of these, only antigen no. 39 exhibits consistently increased expression (about 25%) following disruption of the vesicles by various techniques. It is possible, therefore, that antigen no.39 is a transmembrane protein with most of its antigenic determinants on the outer surface of the membrane.

Antigen no. 28 is unique in that it shows anomalous behavior during adsorption of anti-vesicle serum with $NaDodSO_4$-treated vesicles (Figure 1D). Since this biphasic response is not observed for intact vesicles or for vesicles disrupted either by physical methods or by solubilization with Triton X-100, the phenomenon is not a consequence of the topological arrangement of the molecule in the membrane. A reasonable explanation for the behavior of antigen no. 28 is that treatment of vesicles with $NaDodSO_4$ selectively denatures some, but not all, of its determinants. Consequently, precipitating antibodies directed against this antigen are always present in serum which has been exhaustively adsorbed with $NaDodSO_4$-treated vesicles. The most obvious candidate for an antigen of this type would be a glycoprotein (Bøg-Hansen et al., 1977). However, results of experiments involving crossed immunoaffinoelectrophoresis (Smyth et al., 1976; Owen & Kaback, 1979) seem to rule out this possibility. Alternatively, the

immunoprecipitate representing antigen no. 28 could comprise two or more discrete immunogens, one of which is sensitive to denaturation by $NaDodSO_4$. Evidence, albeit controversial, for the existence of more than one membrane component within some CIE immunoprecipitates has been reported (for a review, see Owen & Smyth, 1977). CIE of ^{125}I-labeled vesicle antigens followed by biochemical analysis of excised regions of immunoprecipitate no. 28 may resolve this question. The observation that antigen no. 26 is expressed to a lower extent in $NaDodSO_4$-dispersed vesicles than in vesicles disrupted by other techniques (cf. Table I) may also reflect a tendency toward partial denaturation. However, it is notable that no other membrane immunogen of this organism or of *Micrococcus lysodeikticus* (Owen, P., unpublished data) appears to be denatured under these conditions.

In conclusion, the distribution of 18 anitgens across the membrane of vesicles prepared from *E. coli* ML 308-225 has been documented, and it seems clear that over 95% of the membrane in these preparations is in the form of sealed vesicles with the same configuration as the membrane in the intact cell. Furthermore, dislocation of any of the antigens tested does not exceed about 11%. While these results are not entirely consistent with certain findings (Van Theinen & Postma, 1973; Hare et al., 1974; Futai, 1974; Weiner, 1974; Futai & Tanaka, 1975; Wickner, 1976; Adler & Rosen, 1977; Yamoto et al., 1978), it is apparent that they confirm and extend a variety of other experimental data (cf., Kaback et al., 1977) demonstrating that membrane vesicle prepared from *E. coli* ML 308-225 by osmotic lysis (Kaback, 1971; Short et al., 1975) retain the topology and polarity of the membrane in the parent bacterium. Reasons for some of the differences are not completely clear, but they may relate to the use of different strains of *E. coli*, to differences in the method of vesicle preparation, or to equivocal interpretation of the techniques utilized (Owen & Kaback, 1978).

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