# Functional *lac* Carrier Proteins in Cytoplasmic Membrane Vesicles Isolated from *Escherichia coli*. 2. Experimental Evidence for a Segregation of the *lac* Carrier Proteins Induced by a Conformational Transition of the Membrane Lipids†

Lucienne Letellier, Rudolf Weil, and Emanuel Shechter*

ABSTRACT: The conformational transition of the lipids in *Escherichia coli* membrane vesicles from a disordered to an ordered state induced by temperature is accompanied by a partial segregation of lipids and proteins in different domains of the membranes. These are domains enriched in lipids that are mainly ordered and domains enriched in proteins that contain mainly disordered lipids. In membrane vesicles that are prepared from K 1059 *E. coli* cells supplemented with linolenic acid, it was possible to isolate defined membrane fractions that correspond to these domains. The amount of *lac* carrier proteins which becomes accessible upon energizing has been determined for linolenate membranes as a function of temperature. It was similarly determined in the fraction that corresponds to the domains containing primarily ordered lipids. The *lac* carrier proteins were titrated by making use of the ability of dansyl galactoside (6′-(*N*-dansyl)aminohexyl 1-thio-β-D-galactopyranoside) to bind to the *lac* carrier with a large increase in its fluorescence. The results confirm previous observations showing the temperature dependence of the amount of *lac* carrier proteins that is involved in the binding of dansyl galactoside and in the transport of β-galactosides. The numerical value decreases with decreasing temperature as a fraction of the lipids become ordered. Furthermore it is demonstrated that, in the temperature range where membrane domains of ordered and disordered lipids coexist, the *lac* carrier proteins segregate preferentially in the disordered domains. In these domains, about half of the *lac* carrier proteins are nonfunctional, while those occupying the ordered domains are functional and participate in the transport process.

In a previous article we have shown that the number of functional *lac* carrier proteins taking part in the transport of β-galactosides in *E. coli* membrane vesicles is temperature dependent (Thérisod et al., 1977). The functional carriers were titrated through the use of the dansyl galactosides. These compounds bind specifically in an energy-dependent way to the carrier proteins with a concomitant large change in fluorescence (Reeves et al., 1973; Schuldiner et al., 1975). We have established that with decreasing temperature there is a decrease in the number of functional *lac* carrier proteins. This decrease parallels the conformational transition of the membrane lipids from a fluid disordered to an ordered state. We have concluded that the low rate of β-galactoside transport usually observed at low temperature is mainly the result of the decrease in the number of functional *lac* carrier proteins rather than the result of an increase in the energy of activation of the overall transport process.

The disorder to order transition of membrane lipids induces the segregation of certain lipids and certain proteins within different areas of the membrane (James and Branton, 1973; Verkleij et al., 1972; Shechter et al., 1974, 1975). In certain instances, this leads to large segregated areas that possess distinctly different physical and chemical characteristics. Accordingly it has been possible to achieve physical separation of the different membrane domains and to isolate different membrane fractions that correspond to these domains (Letellier and Shechter, 1976; Letellier et al., 1977).

In this article we present the results of a study of the binding of dansyl galactoside to a fraction of membrane vesicles which corresponds to a well-defined membrane domain. We compare these results with those of the binding of dansyl galactosides to nonfractionated membranes. The data confirm the conclusions drawn previously on the temperature dependence of the concentration of the functional *lac* carrier proteins (Thérisod et al., 1977). Moreover they extend them in two important respects. First, it is shown that the disorder to order transition of the membrane lipids with temperature is not the direct cause for the change in the number of functional *lac* carrier proteins with temperature; second, it is shown that, during this transition, the *lac* carrier proteins segregate in different domains of the membrane, those domains that contain fluid lipids, and those domains that contain ordered lipids. We present a quantitative estimate of this segregation.

## Materials and Methods

Cells of *E. coli* K 1059 ($i^+ z^+ y^+ a^+$), an unsaturated fatty acid auxotroph, were grown as described previously with linolenic acid (*cis,cis,cis*-$\Delta^{9,12,15}$-$C_{18:3}$) as the exogenous unsaturated fatty acid (Shechter et al., 1974). Membrane vesicles were prepared as described by Kaback (1971) and will be referred to as "linolenate" membrane vesicles. Fractions of these membrane vesicles were obtained with the use of slightly different procedures involving osmotic shock of the spheroplasts at 4 °C rather than at 37 °C (Letellier and Shechter, 1976). In this study the only fraction of interest corresponds to a region of the nonfractionated membrane containing the ordered lipids at low temperature. These will be termed "linolenate light fraction" membrane vesicles.

In this report we refer to membrane vesicles isolated from *E. coli* K 1059 grown on oleic acid (*cis*-$\Delta^9$-$C_{18:1}$) and from *E.*

† From the Laboratoire des Biomembranes (LA 272), Département de Biochimie et de Biophysique, Université de Paris-Sud, 91405 Orsay, France (L.L. and E.S.), and from the Sandoz Forschungsinstitut, Wien, A 1235, Austria (R.W.). *Received February 2, 1977.* This work was supported in part by a grant from the Délégation Générale à la Recherche Scientifique et Technique, Comité des Membranes Biologiques.

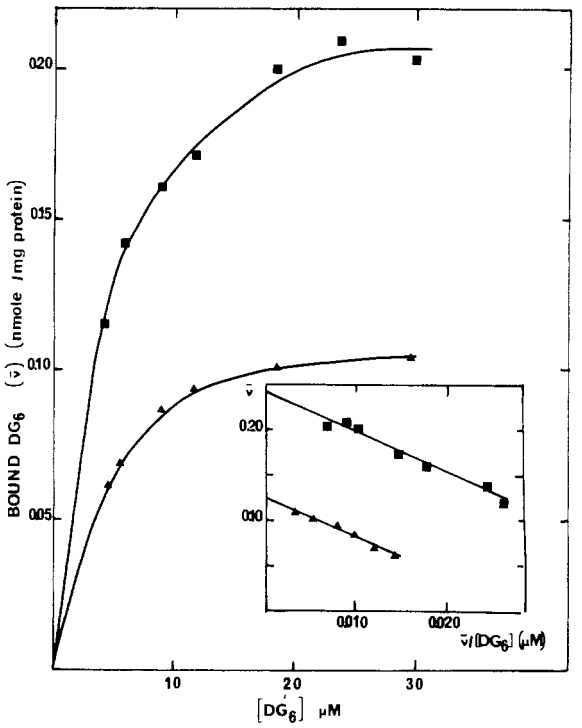


FIGURE 1: "Linolenate" membranes; effect of $DG_6$ concentration on the D-lactate-dependent fluorescence changes at 7 °C (▲) and 40 °C (■). At each temperature, the percentage increase in $DG_6$ fluorescence following D-lactate addition was determined at each $DG_6$ concentration (excitation, 340 nm; emission, 500 nm). All values were corrected by subtracting the intensity of light scattered by the membranes. The number of $DG_6$ molecules bound at each concentration was calculated on the assumption that bound $DG_6$ increased its fluorescence by a factor of 39 (Thérisod et al., 1977). Inset: data plotted according to Scatchard representation. The regression line was calculated by the method of least squares.

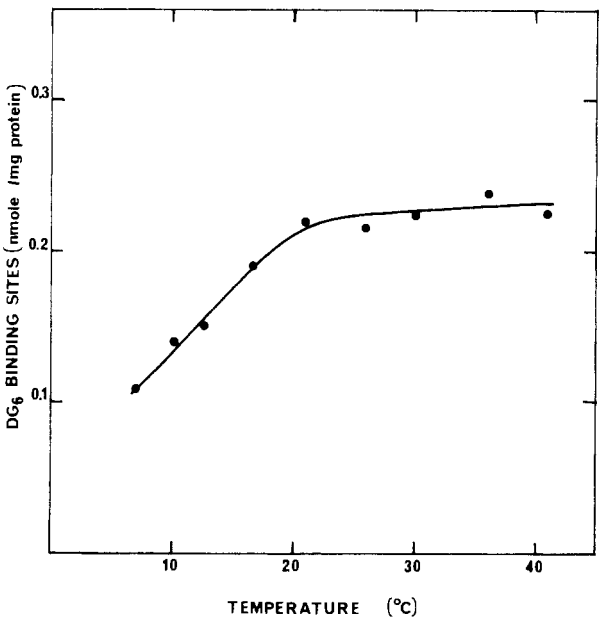


FIGURE 2: "Linolenate" membranes; temperature dependence of the total number of binding sites of $DG_6$ induced by the addition of D-lactate. Qualitatively, a similar temperature dependence of the number of binding sites for $DG_6$ is observed on addition of succinate rather than D-lactate as the electron donor (data not shown).

*coli* ML 308225 as "oleate" and ML 308225 membrane vesicles, respectively. Other techniques such as fluorescence and uptake have been described previously (Thérisod et al., 1977). The synthesis of $DG_6$[1] was performed as described by Schuldiner et al. (1975).

## Results

The emission spectra of $DG_6$ in the presence of "linolenate" and "linolenate light fraction" membrane vesicles display an increase in fluorescence on the addition of D-lactate. This increase in fluorescence is similar to that reported for $DG_6$ in the presence of ML 308225 and "oleate" membrane vesicles (Thérisod et al., 1977; Schuldiner et al., 1975). It shows an emission maximum at 490 nm. This is due specifically to binding to the *lac* carrier protein and results from the transfer of dansyl moiety from the polar water environment to the less polar environment of the membrane.

*Temperature Dependence of the Number of $DG_6$ Binding Sites.* The increase in fluorescence is a saturable function of the external $DG_6$ concentration. The amount of $DG_6$ bound at infinite $DG_6$ concentration can be determined from the saturation curves. This yields the number of binding sites that are equivalent to the number of *lac* carrier proteins accessible to the external medium (Thérisod et al., 1977; Reeves et al., 1973; Schuldiner et al., 1975).

For "linolenate" membrane vesicles the number of $DG_6$ binding sites is temperature dependent. Figure 1 shows the saturation curves at the two extreme temperatures, 7 °C and 40 °C. Figure 2 shows the number of binding sites as a function of temperature as determined from the saturation curves. The number of sites decreases from a constant value of 0.23 nmol/mg protein at temperatures above 25 °C to a low value of 0.12 nmol/mg protein at 7 °C, the lowest temperature investigated. Qualitatively this behavior is similar to that described previously for "oleate" and ML 308225 membrane vesicles (Thérisod et al., 1977). The decrease in the number of $DG_6$ binding sites takes place at the temperature range of the transition of the membrane lipids from the disordered to the ordered state (Shechter et al., 1974; Letellier et al., 1977). Quantitatively, however, the number of binding sites at the high temperatures where all the lipids are disordered, is smaller for "linolenate" membrane vesicles than for "oleate" membrane vesicles (0.60 nmol/mg protein) (Thérisod et al., 1977). This difference may be the result of a variation in the fluidity of the membrane at the growth temperature. Indeed, the cells are grown at 37 °C. At this temperature, a sizeable fraction of the lipids of "linolenate" membranes are still ordered (Shechter et al., 1974; Letellier et al., 1977). This may hinder the synthesis and/or the incorporation of the *lac* carrier proteins into the membrane.

The number of $DG_6$ binding sites for "linolenate light fraction" membrane vesicles is temperature independent. Within experimental error, the number of binding sites is constant: 0.20 ± 0.03 nmol/mg protein (data not shown). This is all the more surprising because "linolenate light fraction" displays a large order-disorder transition of membrane lipids in the temperature range between 20 °C and 40 °C (see Discussion).

The dissociation constant $K_D$ is temperature independent within experimental error and is similar for both "linolenate" and "linolenate light fraction" membrane vesicles: $K_D = 5\ \mu M$. This value is the same as that reported previously for "oleate" and "ML 308225" vesicles (Thérisod et al., 1977; Schuldiner et al., 1975).

*Temperature Dependence of the Initial Rates of $DG_6$ Binding.* The initial rate of $DG_6$ binding calculated from the

[1] Abbreviation used: $DG_6$, 6′-(*N*-dansyl)aminohexyl 1-thio-β-D-galactopyranoside.

linear portion of the curve of the fluorescence increase that is observed on the addition of D-lactate is temperature dependent. This was calculated as a function of temperature at a single concentration of $DG_6$ (10 $\mu$M or $2K_D$).

The data for "linolenate" membrane vesicles are shown in Figure 3 and are plotted according to the Arrhenius equation. If the initial rates are calculated as nmol of $DG_6$ bound per mg of protein per min (left ordinate), the best fit of the experimental points would be represented by two straight lines with a steeper slope in the low temperature range. If on the other hand the initial rates are calculated as nmol of $DG_6$ bound per nmol of binding sites per min (right ordinate), the best fit comprises only one line with a slope similar to that obtained in the higher temperature range of the first representation.

The data for "linolenate light fraction" vesicles are also shown in Figure 3. The experimental values of both types of representation discussed above (nmol of $DG_6$ bound per mg of membrane protein (left ordinate) or per nmol of binding sites (right ordinate)) can best be fitted on only one line. The slope of this line is similar to that obtained in the Arrhenius plot of "linolenate" membrane vesicles in the high temperature range.

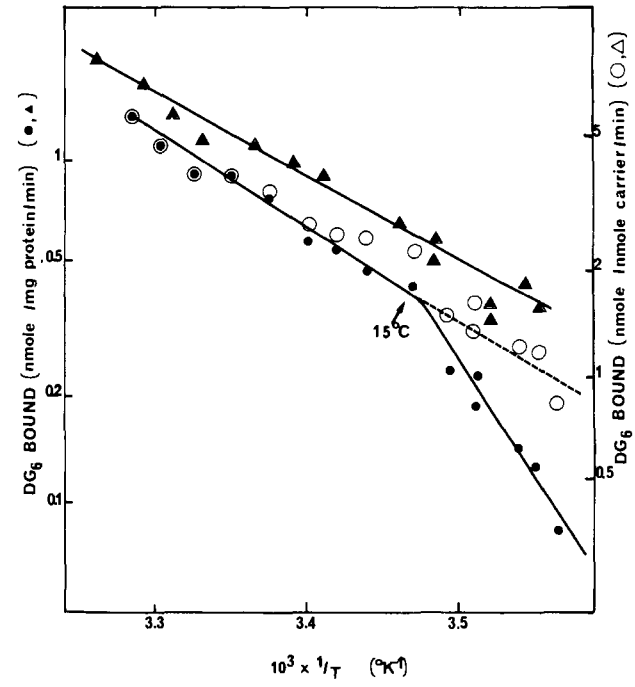


FIGURE 3: Temperature dependence of the initial rates of binding of $DG_6$ by "linolenate" and "linolenate light fraction" membranes. The initial rates of binding were calculated from the linear portion of the fluorescence increase induced by the addition of D-lactate. Left ordinate: initial rates expressed as nmol of $DG_6$ bound per mg of protein per min for "linolenate" (●) and "linolenate light fraction" (▲) membranes, respectively. Right ordinate: initial rates expressed as nmol of $DG_6$ bound per nmol of carrier per min for "linolenate" (○) and "linolenate light fraction" (△) membranes, respectively. The number of functional carriers, equivalent to the number of binding sites (Thérisod et al., 1977; Schuldiner et al., 1975), is taken from Figure 2. For "linolenate" membranes, the total number of binding sites is constant at temperatures above 25 °C and the two representations lead to parallel curves that are displaced by a constant quantity. The two ordinates are displaced by this quantity, and thus the experimental points above 25 °C are common to both representations. For "linolenate light fraction" membranes, the number of binding sites is temperature independent, and thus the experimental points are common to both representations over the entire temperature range.

## Discussion

We have shown in the previous article that there exists for "oleate" and ML 308225 membrane vesicles a striking parallel between the temperature dependence of the initial rates of $DG_6$ binding and of the initial rates of $\beta$-galactoside transport. This was taken as an indication that the carriers which effectively participate in transport (the functional carriers) are those which become accessible to $DG_6$ binding upon energizing.

Both "linolenate" and "linolenate light fraction" membrane vesicles transport $\beta$-galactosides to the same extent as those of "oleate" and ML 308225 (approximately 20 nmol per nmol of functional *lac* carrier protein per min at 25 °C (data not shown)). Because of the small number of *lac* carrier proteins present in "linolenate" membrane, it was not possible to determine the complete temperature dependence of the initial rates of $\beta$-galactoside transport. It was only possible to determine the temperature dependence of the initial rates of $DG_6$ binding.

The results presented here on "linolenate" membrane vesicles confirm previous conclusions drawn from the study of "oleate" and ML 308225 membrane vesicles (Thérisod et al., 1977). These are: first, that the break and increased slope observed in the low temperature range of the usual Arrhenius plots of $\beta$-galactoside transport are mainly if not totally a consequence of the decrease in the number of functional *lac* carrier proteins taking part in transport in this temperature range; second, that this decrease is the result of the existence of a transition of the membrane lipids from a disordered to an ordered state with decreasing temperature.

Furthermore, the results obtained on "linolenate light fraction" membrane vesicles demonstrate that this transition is not the direct cause for the decrease in the number of functional carriers taking part in transport. Finally a comparison of the results obtained on "linolenate" and "linolenate light fraction" allows a direct determination of the segregation of the *lac* carrier proteins in the different membrane domains during the transition of the lipids from a disordered to an ordered state.

The existence of this phenomenon of segregation of the *lac* carrier proteins has been inferred by Overath et al. (1976) on the basis of $\beta$-galactoside transport in *E. coli* cells. Their calculations are based on a certain number of assumptions, one of which stipulates that the transition does not act on the effective concentration of the carrier taking part in transport. Clearly this assumption is not supported by our experimental data in the case of membrane vesicles. The segregation of the *lac* carrier proteins in different membrane domains can be determined directly and without any assumptions by a comparison of the properties of "linolenate" and "linolenate light fraction" membranes.

Following the nomenclature of Overath et al. (1976), we shall use $F_{tot}$ to represent a given total area of "linolenate" membrane. At temperatures below those of the order-disorder transition of the lipids, this area can be divided into $F_{ord}$ and $F_{fl}$ and $F_{tot} = F_{ord} + F_{fl}$. The fraction of "linolenate" membrane containing the ordered lipids corresponds to the "linolenate light fraction". We call $P_{tot}$ the total amount of *lac* carrier present in "linolenate" membrane. During the disorder to order transition of the lipids, the *lac* carrier might segregate. Similarly, $P_{ord}$ and $P_{fl}$ represent the fractions of the *lac* carrier present in the ordered and fluid domains respectively. $P_{tot} = P_{ord} + P_{fl}$, and $k$ would be a segregation factor defined by $k = (P_{fl}/F_{fl})/(P_{ord}/F_{ord})$. A value of $k$ greater than 1 denotes a preferential segregation of the carrier into membrane domains containing fluid lipids while a value smaller than 1 denotes a preferential segregation in domains containing ordered lipids.

The details of the calculation are developed in the legend of Figure 4. This figure represents a highly schematic illustration

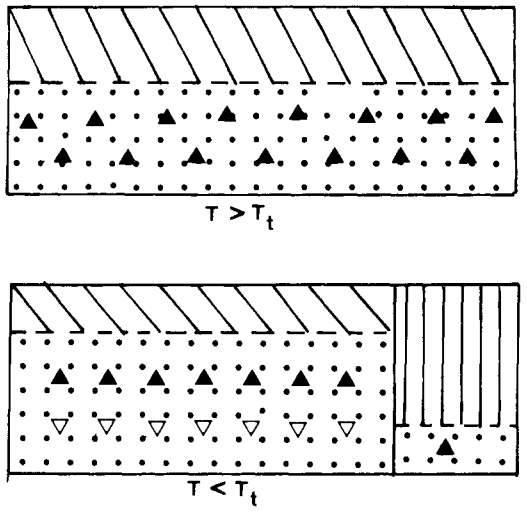


FIGURE 4: "Linolenate" membranes; schematic distribution of lipids, proteins, and *lac* carrier proteins at temperatures above ($T > T_t$) and below ($T < T_t$) those of the order-disorder transition of membrane lipids. The hatched and the dotted regions represent the relative membrane areas occupied by lipids and proteins, respectively. Diagonal and vertical hatchings represent disordered and ordered lipids, respectively. Closed and open triangles represent the functional and nonfunctional *lac* carrier proteins. At temperatures above the transition temperature ($T > T_t$), all lipids are disordered. The relative composition of "linolenate" membranes is 40% lipids and 60% proteins (Letellier and Shechter, 1976; Letellier et al., 1977). The relative area occupied by the lipids and the proteins is 0.4 and 0.48, respectively (we assume a density of 1 for the lipids and of 1.25 for the proteins). At temperatures below transition temperature ($T < T_t$), 40% of the total lipid become ordered (Shechter et al., 1974). These lipids which form 16% of the total mass are confined in the ordered domains. These domains correspond to the "linolenate light fraction" membranes which have been isolated and whose characteristics have been determined independently (Letellier and Shechter, 1976; Letellier et al., 1977). "Linolenate light fraction" membranes are enriched in lipids, and the relative composition is 70% lipids and 30% proteins (Letellier and Shechter, 1976; Letellier et al., 1977). Thus the proteins in the ordered domains make up (3/7) × 16% = 6.85% of the total membrane mass. The ordered domains occupy a membrane surface equal to 16%/100 + 6.85%/125 = 0.215. The total membrane surface being equal to 0.4 + 0.48 = 0.88, the fluid domains occupy a surface equal to 0.88 − 0.215 = 0.665. The ratio $F_{ord}/F_{fl}$ = 0.215/0.665 = 0.32. The ordered domains contain 6.85%/0.6 = 11.4% of the total membrane proteins. "Linolenate" and "linolenate light fraction" membranes have 0.23 and 0.20 nmol of *lac* carrier proteins per mg of membrane protein. Thus the fraction of carriers in the ordered domains is (0.2/0.23) × 0.114 = 0.10. The fraction of carrier in the fluid domains is 0.9. The ratio $P_{fl}/P_{ord}$ = 9. The segregation factor $k$ for the *lac* carrier proteins is 2.9.

of the distribution of lipids, proteins, and *lac* carrier proteins between the different membrane domains. The calculation leads to a value of $k = 2.9$ and indicates a preferential segregation of the *lac* carriers into the membrane domain containing the fluid lipids.

*E. coli* membrane vesicles catalyze the active transport of a variety of metabolites other than β-galactosides. These transports are also coupled to the oxidation of D-lactate (Kaback, 1974). The metabolites are transported through a series of specific membrane carriers. We have indicated that the properties of β-galactoside and proline transport are similar (Thérisod et al., 1977). Thus, some other specific carriers may display the same preferential segregation into the membrane domains containing the fluid lipids.

The decrease in the number of functional *lac* carrier proteins occurs concomitantly with a transition of the membrane lipids as we noted earlier with "oleate" and ML 308225 membranes (Thérisod et al., 1977) and "linolenate" membranes described here. However, these transitions do not involve an obligatory decrease in the number of functional carrier. "Linolenate light fraction" membranes display a large order-disorder transition between 20 and 40 °C in which practically all the lipids participate (Letellier et al., 1977); yet the amount of carrier binding $DG_6$ is temperature independent. This indicates that the carriers present in the ordered domains of "linolenate" membranes are functional and participate in the transport process. In fact, even the affinity of the carriers for a given β-galactoside is temperature independent (5 μM for $DG_6$). It follows that the carriers which do not participate anymore in transport are present in the membrane domains containing the fluid lipids.

This conclusion stems also from the observation that less than 10% of the carriers of "linolenate" membrane are situated in the ordered domains while more than 50% do not participate further in transport (see Figure 4).

The exact reason for the existence of two categories of carriers in the fluid regions, the functionals and the nonfunctionals, is still unclear. It may result from a physical inaccessibility of certain carriers to the external medium. Indeed, the fluid regions are enriched in proteins (Letellier and Shechter, 1976; Letellier et al., 1977) and as visualized by freeze-fracture electron microscopy, these proteins tend to aggregate (Shechter et al., 1974). On the other hand, the existence of nonfunctional carriers may result from a segregation into different regions of the membrane of different components of the same transport system. In this respect, it has been reported that the D-lactate dehydrogenase, the first enzyme involved in the oxidation of D-lactate, segregates preferentially during the transition into the ordered domain of the membrane (van Heerickhuisen et al., 1975).

These and other possibilities are currently being investigated.

Acknowledgments

We would like to thank Dr. V. Najjar for correcting the manuscript.

References

James, R., and Branton, D. (1973), *Biochim. Biophys. Acta 323*, 378–390.
Kaback, H. R. (1971), *Methods Enzymol. 22*, 99–120.
Kaback, H. R. (1974), *Science 186*, 882–892.
Letellier, L., Moudden, H., and Shechter, E. (1977), *Proc. Natl. Acad. Sci. U.S.A. 74*, 452–456.
Letellier, L., and Shechter, E. (1976), *J. Microsc. Biol. Cell. 25*, 191–196.
Overath, P., Thilo, L., and Träuble, H. (1976), *T.I.B.S. 1*, 186–189.
Reeves, J. P., Shechter, E., Weil, R., and Kaback, H. R. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70*, 2722–2726.
Schuldiner, S., Kerwar, G. K., Weil, R., and Kaback, H. R. (1975), *J. Biol. Chem. 250*, 1361–1370.
Shechter, E., Letellier, L., and Gulik-Krzywicki, T. (1974), *Eur. J. Biochem. 49*, 61–76.
Shechter, E., Letellier, L., and Gulik-Krzywicki, T. (1975), in Molecular Aspects of Membrane Phenomena, Kaback, H. R., Neurath, H., Radda, G. K., Schwyzer, R., and Wiley, W. R., Ed.; Berlin, Springer-Verlag, pp 39–63.
Thérisod, H., Letellier, L., Weil, R., and Shechter, E. (1977), *Biochemistry 16* (preceding paper in this issue).
van Heerickhuisen, H., Kwack, E., van Bruggen, E. F. J., and Witholt, B. (1975), *Biochim. Biophys. Acta 413*, 177–191.
Verkleij, A. J., Ververgaert, P. H. J. Th., van Deenen, L. L. M., and Elbers, P. F. (1972), *Biochim. Biophys. Acta 288*, 326–332.