

Lateral Phase Separations in Membrane Lipids and the Mechanism of Sugar Transport in *Escherichia coli*

(β -oxidationless fatty-acid auxotrophs/spin labels)

C. D. LINDEN†, K. L. WRIGHT§, H. M. McCONNELL§, AND C. F. FOX†

† Department of Bacteriology and Molecular Biology Institute, University of California, Los Angeles, Calif. 90024; and
§ Stauffer Laboratory for Physical Chemistry, Stanford, California 94305

Contributed by Harden M. McConnell, May 7, 1973

ABSTRACT Changes in slope of Arrhenius plots for transport can, in some instances, be detected at two different temperatures for cells that have a relatively simple fatty-acid composition in the membrane lipids. These characteristic temperatures correlate with the characteristic temperatures that define changes of state in membrane phospholipids as revealed by the paramagnetic resonance of the spin label TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl). The higher of these characteristic temperatures is that at which the formation of solid patches of membrane lipids is first detected. The lower is the end point of the course of lateral phase separations, at which all the membrane lipids are in a solid phase. For cells enriched for elaidic acid, the rate of transport increases by as much as 2-fold as the temperature is decreased by less than 1°, at the higher characteristic temperature. At this characteristic temperature, lateral phase separations begin in the membrane phospholipids. This is also the temperature where one predicts a striking increase in the lateral compressibility of the membrane lipids. These data are thus interpreted to indicate that a component of the transport system vertically penetrates one or both monolayer faces of the membrane during transport, or that some other event involving the lateral compression of the phospholipids is important for transport.

Essential fatty-acid auxotrophs of *Escherichia coli* are powerful tools that have been exploited to study the influence of lipid physical properties on the function and assembly of cellular membranes (1-7). Arrhenius plots for transport have been shown to have a biphasic shape, and the characteristic temperature that defines the change in slope in these plots is exquisitely responsive to the physical characteristics of the essential fatty-acid supplement present during cellular growth. The characteristic temperatures reflect the melting characteristics of the essential fatty-acid supplement, e.g., the characteristic temperatures are in decreasing order when determined in cells grown in media supplemented with fatty acids that are *trans*-monoenoic, *cis*-monoenoic, and *cis*, *cis*-dienoic (or *cis*, *cis*, *cis*-trienoic), (2-4, 6). The first strong indications that the characteristic temperatures correspond to a change of state in the membrane lipids came from two independent studies: (1) The characteristic temperatures for two unrelated transport systems were identical for cells grown with a single essential fatty acid (2, 4). (2) A change in state detected in a monolayer of phosphatidyletha-

Abbreviations: T is the absolute temperature (degrees Kelvin), t_h and t_l refer to "higher" and "lower" characteristic temperatures (°C) as revealed by spin labeling (or other physical techniques), and t_h^* and t_l^* refer to the higher and lower temperatures (°C) as revealed by transport rate. The spin label TEMPO is 2,2,6,6-tetramethylpiperidine-1-oxyl.

nolamine (the major phospholipid in *E. coli*) correlated with the characteristic temperature for transport (3). Neither study, however, led to a clear physical interpretation. In the present report, we describe experimental data that show that transport induced in a fatty-acid auxotroph grown with a single essential fatty-acid supplement may have not one, but two characteristic temperatures, and that these correspond to the upper and lower temperature boundaries of the course of lateral phase separations in the membrane lipids.

Lateral phase separations in membranes are distinct from "phase transitions" that occur in pure (one-component) lipid membranes. In the former case, extensive lateral relative motion (lateral diffusion) of membrane components is required, whereas in the latter case, little or no relative motion is required (8). Rapid lateral motion of spin-labeled lipids has been measured in model membranes (lipid bilayers) (8-10), and in intact, functional biological membranes (11, 12). Finally, the temperature-composition *phase diagrams* for lateral phase separations of two-component pure lipid systems can be determined by use of spin-label techniques (13, 14) as well as calorimetric methods (15). Freeze-fracture electron micrographs of binary phospholipid mixtures (16, 17) and biological membranes (18-20) sometimes show zones and/or "particle free" patches that may be due to lateral phase separations of the type considered here. It will clearly be of considerable interest to perform such studies on the *E. coli* membranes investigated in the present study.

MATERIALS AND METHODS

Growth and Properties of Bacterial Strains. Strain 30E β ox⁻ was used exclusively in the studies reported here. It is an unsaturated fatty-acid auxotroph of *E. coli* K12, defective in the β -oxidation of fatty acids. The properties of the parent strain 30E have been described (21, 22). Cells were cultured in a medium consisting of medium A (23) supplemented with 1% Difco casamino acids, 5 μ g/ml of thiamine HCl, 0.5% of the nonionic detergent Triton X-100 (Rohm and Haas), and 0.02% of an essential fatty acid. Elaidic (*trans*-9-octadecenoic), oleic (*cis*-9-octadecenoic), and linoleic (*cis*, *cis*-9,12-octadecadienoic) acids were purchased from the Hormel Institute, Austin, Minn. Cultures of 500 ml were grown with vigorous rotary agitation at 37° in 2-liter flasks. Before they were harvested, the cells were grown with the indicated essential fatty acid for at least five doublings of cell mass, and growth was followed turbidimetrically.

Strain 30E β ox⁻ was isolated from the parent strain by the following procedure. Cells of strain 30E were grown in medium consisting of medium A (22), 0.4% succinic acid (neutralized to

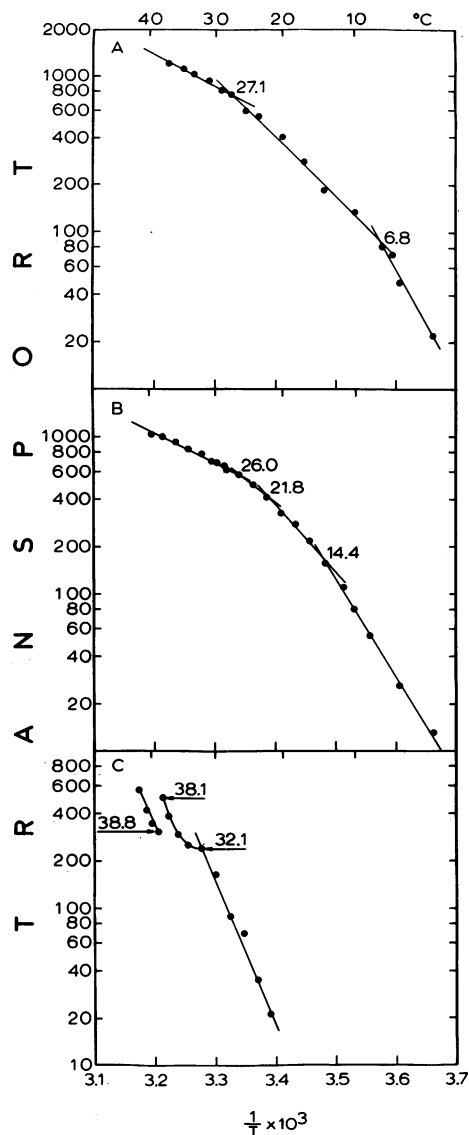


FIG. 1. Arrhenius plots for β -glucoside transport by cells grown at 37° in medium supplemented with (A) linoleic acid, (B) oleic acid, and (C) elaidic acid. The data in parts (A) and (B) were analyzed to assess the best plots for all contiguous points considered in groups of three or more. We consider the upper two intersects (26.0° and 21.8°) in part (B) to be a creation of the computer program and attach to them no physical or biological significance. The units for transport of *p*-nitrophenyl- β -glucoside are nmol/20 min per 2×10^9 cells.

pH 7 with KOH), 0.02% oleic acid, 0.5% of Triton X-100, and 5 μ g/ml of thiamine HCl. An aliquot of cells was subjected to nitrosoguanidine mutagenesis (24). Mutagenized cells were grown for an additional 10 generations in the same medium and then treated for 20 hr with 300 units of penicillin per ml in this same medium, modified to contain no succinic acid. Survivors were plated on the initial growth medium containing 1.5% agar, and screened for mutants defective in β -oxidation by replica plating onto solid medium containing oleic acid, but no succinic acid. Strain 30E β ox⁻ is defective in β -oxidation by two criteria: (1) The extent of release of ¹⁴CO₂ from a carboxyl-labeled fatty acid was over 100-fold less in the mutant than in the parent strain (Table 1), and (2) virtually no β -

oxidation products derived from exogenously supplied essential fatty acids were detected by analysis of methyl esters of the fatty acids derived from phospholipids of the mutant strain (Table 2).

Induction and Assay of Transport. For induction of β -glucoside transport, salicin (Aldrich Chemical Co.) was included in the medium at 0.1% for at least four generations of growth. For induction of β -galactoside transport, 0.2 mM isopropyl-1-thio- β -galactopyranoside was included in the medium for the final hour of growth. The transport assay procedures have been described in detail (4).

Isolation of *E. coli* Inner Membranes. Cells were grown as described to a density of 10⁹ cells per ml. At this point, an aliquot was removed and processed for assay of transport (4). The remaining cells were collected by centrifugation and washed to remove detergent by suspension in medium containing no fatty acid or casamino acids. They were then converted to spheroplasts, lysed, and treated to resolve the "inner" (cytoplasmic) and "outer" membrane fractions (22, 26). The sole modification in procedure was elimination of 2-mercaptoethanol since this compound can reduce nitroxides. The separation of inner and outer membranes was monitored by assay of appropriate enzyme markers, succinate dehydrogenase for inner membranes (27) and phospholipase A for outer membranes (28). The inner membrane preparations used here contained a maximum of 15% outer membrane contamination. Protein was determined by the method of Lowry *et al.* (29).

Extraction and Characterization of Lipids. Lipids were extracted from membranes with 2:1 chloroform-methanol as described (30). Fatty-acid methyl esters were obtained by transesterification with BF₃-methanol (Applied Science Laboratories), and the methyl esters were separated by gas-liquid chromatography on a 6 ft \times 1/8 in column of 10% EGSSX on Chrom W DMCS.

Spin Labels. The use of the spin label TEMPO (2,2,6,6-

TABLE 1. Comparison of the extent of fatty-acid β -oxidation mediated by strain 30E β ox⁻ and the parent strain 30E

cpm ¹⁴ CO ₂ released per cpm in cellular lipids	
30E β ox ⁻	0.48
30E	51.36

Cells of strains 30E and 30E β ox⁻ were grown in exponential phase in medium supplemented with oleic acid, harvested, washed, and suspended at a density of 5×10^8 cells per ml in medium A (22) containing 0.5% Triton X-100. A 5-ml portion of each cell suspension was introduced outside the center wells of 125-ml center well erlenmeyer flasks. Each center well contained 1 ml of 0.1 M Tris base and a pleated strip of filter paper. 1 μ Ci of carboxyl-labeled [¹⁴C]linoleic acid (New England Nuclear Corp., 4 Ci/mol) in 0.1 ml of ethanol was added to each cell suspension. The flasks were sealed and incubated for 6 hr at 37° with gentle shaking. An aliquot of cells was then quantitatively removed from the flasks and washed extensively on a Millipore HA filter with medium containing detergent. Both the cells and the contents of the center wells were then transferred to vials containing the solution described by Patterson and Greene (25) for scintillation counting. The ratio of radioactivity collected in the center well to radioactivity in cellular lipid was calculated from these data.

TABLE 2. Fatty-acid composition of inner membrane phospholipids

Fatty acid	30E		30E β ox ⁻	
	Oleate (%)	Oleate (%)	Linoleate (%)	Elaidate (%)
14:0	4.6	6.5	2.9	4.5
16:0	42.1	38.5	44.0	8.7
16:1(<i>cis</i>)	16.9	2.0	1.9	—
18:1(<i>cis</i>)	36.5	51.0	—	—
18:1(<i>trans</i>)	—	—	—	86.8
18:2(<i>cis,cis</i>)	—	—	48.9	—
Other	1.0	2.0	2.3	1.0
Saturated/Unsaturated	0.87	0.85	0.92	0.15

Details are described in *Methods*.

tetramethylpiperidine-1-oxyl) to study lateral phase separations in lipid mixtures and in biological membranes is described in detail elsewhere (13, 14). Chemical reduction of TEMPO was prevented by thorough washing of the membranes.

RESULTS

Arrhenius plots for β -glucoside transport by cells grown in media supplemented with (A) linoleic, (B) oleic, and (C) elaidic acids are presented in Fig. 1. In each case there is a clear lower characteristic temperature. These lower characteristic temperatures are in excellent agreement with those reported by Wilson, Rose, and Fox (2) and by Wilson and Fox (4) using the parent strain of that used here. In addition, there are in parts (A) and (C) clear upper characteristic temperatures. At the upper characteristic temperature in (C) there is a nearly 2-fold increase in transport rate where the temperature for transport assay is decreased by less than 1° (from 38.8° to 38.1°). In an independent experiment identical to that described in Fig. 1C, a superimposable plot was obtained and the dramatic increase in transport rate with decreasing temperature was observed between 39.1° and 38.6°. Combining these two experiments, it appears that there is a very sharp upward discontinuity in the plot for transport rate between 38.6° and 38.8°. The curve in (C) assumes a parabolic appearance between 38.1° and 32.1°, and as the transport assay temperature approaches 32°, the activation energy of transport approaches zero. At about 32°, there is an abrupt increase in the activation energy for transport. The greater detail revealed in Fig. 1C, over that revealed in Fig. 1A and B may be a consequence of lipid composition. The phospholipids of elaidate-grown cells approximate a binary mixture on the basis of fatty-acid composition (31). Diglyceride analysis shows that the fatty-acid distribution in phospholipids derived from oleate- or linoleate-grown cells is far more complex.

TABLE 3. Characteristic temperatures for glucoside transport (t_h^* , t_l^*) and TEMPO partitioning (t_h , t_l)

	t_h^*	t_h	t_l^*	t_l
Elaidic acid	38.6 – 38.8	37.7	32.1	30.7
Oleic acid	26.0 and 21.8	31	14.4	15.8
Linoleic acid	27.1	28.5	6.8	8.9

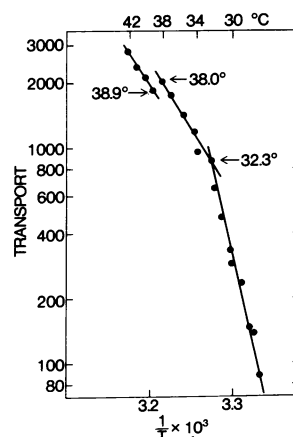


FIG. 2. Arrhenius plot for β -galactoside transport by cells grown at 37° in medium supplemented with elaidic acid. The units for transport of *o*-nitrophenyl- β -galactoside are nmol/20 min per 1×10^9 cells.

The sharp discontinuity observed for β -glucoside transport between 38° and 39° in Fig. 1C is also a characteristic of β -galactoside transport by cells grown in medium supplemented with elaidic acid (Fig. 2). The plot for β -galactoside transport reveals an intersect at about 32°, but the departure from linear slope between 38° and 32° is less prominent than for β -glucoside transport.

Fig. 3 shows a plot of the TEMPO spectral parameter f against $1/T$ for the inner membranes obtained from the same batch of cells used to obtain the transport data in Fig. 1. This parameter is measured directly from the observed spectra, and is approximately equal to the fraction of the molecules of TEMPO that is dissolved in the fluid hydrophobic region of the membrane (13, 14). The logarithmic plots of f against $1/T$ for the inner membranes derived from 30E β ox⁻ show in each case two well-defined breaks, defining the characteristic temperatures t_l and t_h . These temperatures are compared with

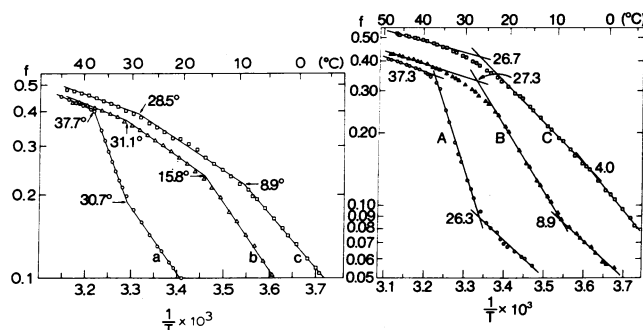


FIG. 3 (left). The TEMPO spectral parameter f as a function of the reciprocal of the absolute temperature ($1/T$) for the inner membranes of 30E β ox⁻ cells derived from the same batch as used to obtain the glucoside transport data in Fig. 1. The cells were grown at 37° in media supplemented with (a) elaidic acid, (b) oleic acid, and (c) linoleic acid. The spectral parameter f is an approximate measure of the solubility of the spin-label TEMPO in the fluid region of the membrane lipids. For a detailed discussion, see Shimshick and McConnell (13, 14).

FIG. 4 (right). The TEMPO spectral parameter f as a function of the reciprocal of the absolute temperature ($1/T$) for aqueous dispersions of phospholipids extracted from the inner membranes of 30E β ox⁻ *E. coli* cells grown at 37° and supplemented with (A) elaidic acid, (B) oleic acid, and (C) linoleic acid.

those obtained from the glucoside transport in Table 3. There is good, but not perfect, agreement between the temperatures t_h and t_h^* , and between t_l and t_l^* . It has been shown elsewhere that breaks in plots of f against $1/T$ can be used to determine the temperature-composition phase diagrams of binary mixtures of pure lipids, and in the cases of relatively simple (solid solution, liquid solution) phase diagrams, the breaks corresponding to the temperatures t_h and t_l measure points on the *liquidus* and *solidus* temperature-composition curves, respectively (13, 14).

Fig. 4 shows a plot of the TEMPO spectral parameter f against $1/T$ for the phospholipids extracted from the inner membranes of 30E β ox⁻ cells grown under the same conditions as those used to obtain the data in Fig. 3 for the inner membranes themselves. It will be seen that the temperatures t_h corresponding to the onset of the phase separations are approximately the same for extracted phospholipids and inner membranes for cells grown on each of the three exogenous unsaturated fatty acids. These temperatures are clearly determined largely by the lipid compositions of the inner membranes. The temperatures t_l corresponding to the completion of the lateral phase separations for extracted lipids and membranes are nearly the same for elaidic acid-grown cells (30.7° and 26.3°), but disagree significantly in the cases of the oleic acid-grown cells (8.9° and 15.8°) and the linoleic acid-grown cells (8.9° and about 4°). These latter discrepancies could arise, for example, from a preferential binding of the more fluid lipids to membrane proteins during lateral phase separations, thus modifying the end-point for this process (t_l).

Based on a comparison of data such as those given in Figs. 3 and 4 with studies of pure binary mixtures (13, 14), it is very likely that the temperatures t_h and t_l do in fact correspond to the onset and completion of lateral phase separations of the phospholipids in the bacterial membrane. If we assume that this conclusion is correct, there are various reasons why the temperatures t_h , t_h^* and t_l , t_l^* may not be in perfect agreement. For example, solvent conditions (ionic strength, pH, and osmolarity) are not identical in the transport and spin-label experiments. Secondly, although it is possible to define the temperatures t_h and t_l in a thermodynamic sense even for a complex mixture of lipids (see *Discussion*), it may be difficult or practically impossible to measure these temperatures with precision experimentally.

DISCUSSION

Pure phospholipids are known to exhibit sharp phase transitions. Binary mixtures of phospholipids, on the other hand, usually exhibit broadened thermal transitions which are nevertheless characterized by a distinct beginning and end. The details of the quantitative aspects of thermal transitions in binary lipid mixtures have been treated elsewhere (13-15); quantitative descriptions require the use of phase diagrams. Mixtures of lipids containing more than two components may likewise be characterized by two temperatures that define the beginning and the end of the process of lateral phase separations.

We have shown that there are upper and lower characteristic temperatures for transport (t_h^* and t_l^*), corresponding to the upper and lower temperatures (t_h and t_l) that define the upper and lower boundaries of the course of lateral phase separations as revealed by the TEMPO spectral parameter.

In earlier studies, correlations between transport and physical transitions in the membrane lipids have been attempted by use of phospholipid monolayers (3) or fluorescence measurements (32). In examining these studies, we are led to the conclusion that in some cases the observed correlations have been between t_h and t_h^* and in other cases t_l and t_l^* , but not both. An examination of the x-ray diffraction data of Esfahani *et al.* (6) reveals that the upper and lower boundaries of the course of the Engelman transition (33) for membranes derived from oleate- and elaidate-grown cells correspond well with the t_h and t_l values reported here.

One of the most interesting results of the present work is the spectacular temperature dependence of the rates of both β -glucoside and β -galactoside transport in elaidate-grown cells. We offer the following explanation for this temperature dependence[†].

Assume that the rate-limiting step for sugar transport involves the vertical penetration of a component of the transport system through the lipid bilayer of the membrane, and that the rate constant for this process has the following form:

$$\text{rate constant} = \nu e^{-\Delta H^\ddagger/RT}$$

Here, ν is a kinetic frequency factor, and ΔH^\ddagger is the activation energy for insertion of the transport-associated protein into the bilayer. In the case of both glucosides and galactosides there is an essentially discontinuous increase in the rate of transport at the higher characteristic temperature t_h^* about 38° as the temperature is reduced. Examination of the log (transport) against $1/T$ plots in Figs. 1 and 2 show that the apparent activation energies ΔH^\ddagger are the same just below and just above t_h^* . This indicates that the discontinuous increase in transport with decreasing temperature is due to an abrupt increase in the frequency factor ν . This abrupt increase in the frequency factor can be understood as follows.

The temperature t_h^* is close or equal to the temperature t_h for the onset of lateral phase separations in the plane of the membrane. At this temperature one predicts on thermodynamic grounds that there is a sudden increase in the isothermal lateral compressibility of the membrane lipids. This enhancement of lateral compressibility then facilitates the insertion of the transport protein. It is known from the fluctuation theory of liquids and gases that high isothermal compressibility is associated with large density fluctuations (34), and we believe that it is these enhanced fluctuations in the packing or density of the membrane lipids that facilitate the insertion of a component of the transport system. It is left for future work to ascertain the spatial as well as the frequency distribution of these density fluctuations[†].

Towards the end of the lateral phase separation in the elaidic acid-grown membranes, the activation energy ΔH^\ddagger for glucoside transport approaches zero. The lipids in these remaining fluid domains are doubtless enriched in chemical

[†] This particular explanation for the enhanced transport is **not** unique. However, all of the other plausible explanations we have considered also depend on the enhanced lateral compressibility of the membrane lipids at t_h . Thus, enhanced lateral compressibility could facilitate an expansion-contraction cycle of a "buried" transport protein. Further, the number of functional transport proteins buried within the bilayer might also be increased by this enhanced compressibility.

composition so that they are lower melting; i.e., they are intrinsically more "fluid" than are the lipids that form the more solid phase. The energy ΔH^\ddagger required for insertion of the glucoside transport protein into this remaining fluid phase at temperatures below t_h and slightly above t_l may well be small, accounting for the low apparent activation energy at this temperature. At temperatures below the temperature t_l where the phase separation is complete (all or nearly all of the lipids are in the solid phase), the activation energy for transport is again large.

Independent studies on the effects of temperature on membrane assembly are consistent with the above discussion (35). Below t_h , membrane assembly processes that must involve insertion of proteins into or through the membrane bilayer become abortive. Thus, processes such as transport and membrane assembly, which involve vertical penetration of membrane bilayers, have a requirement or preference for the *capacity* for lateral compressibility of the membrane lipids. This may be contrasted to the effects of lipid composition on the activity of various membrane-associated enzymes. Though these enzymes are responsive to lipid composition, they do not respond at either the beginning or end of lateral phase separations (6, 36).

Quite recent studies of the rates of permeation of single-component lipid bilayers (model membranes) by ions and molecules also show enhanced rates when two phases are present simultaneously, i.e., when the temperature corresponds to the center of the "phase transition." Thus, Papahadjopoulos *et al.* (37) have observed enhanced rates of self-diffusion of $^{22}\text{Na}^{+-}$ as well as ^{14}C sucrose through dipalmitoylphosphatidyl choline liposomes in the vicinity of 41° , the temperature of the phase transition of this phospholipid (15). Similarly, Wu and McConnell have observed an enhanced valinomycin-mediated K^+ electrical conductivity through a fritted-glass filter containing dipalmitoylphosphatidyl choline at about 41° (38). We believe that the enhanced bilayer permeations are also related to the enhanced lateral compressibility of these model membranes when the fluid and solid lipid phases are in equilibrium with one another.

We are greatly indebted to Mr. Wolfgang Kleemann for obtaining the data given in Fig. 4, and to Mr. Edward Shimshick for many helpful discussions. C.D.L. is a USPHS predoctoral trainee supported by Grant GM-1531. C.F.F. is the recipient of USPHS Research Career Development Award GM 42359. Supported in part by research grants from USPHS (GM 18233, AI 10733), the American Cancer Society (BC-79), the Damon Runyon Fund (DRG-1153), and the National Science Foundation (GB-33501X1).

1. Schairer, H. U. & Overath, P. (1969) *J. Mol. Biol.* **44**, 209-214.
2. Wilson, G., Rose, S. & Fox, C. F. (1970) *Biochem. Biophys. Res. Commun.* **38**, 617-623.
3. Overath, P., Schairer, H. U. & Stoffel, W. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 606-612.
4. Wilson, G. & Fox, C. F. (1971) *J. Mol. Biol.* **55**, 49-60.
5. Overath, P., Hill, F. F. & Lamnek-Hirsch, I. (1971) *Nature New Biol.* **234**, 264-267.

6. Esfahani, M., Limbrick, A. R., Knutton, S., Oka, T. & Wakil, S. (1971) *Proc. Nat. Acad. Sci. USA* **68**, 3180-3184.
7. Fox, C. F. & Tsukagoshi, N. (1972) in *Membrane Research*, ed. Fox, C. F. (Academic Press, New York), pp. 145-151.
8. McConnell, H. M., Devaux, P. & Scandella, C. (1972) in *Membrane Research*, ed. Fox, C. F. (Academic Press, New York), pp. 27-37.
9. Devaux, P. & McConnell, H. M. (1972) *J. Amer. Chem. Soc.* **94**, 4475-4481.
10. Sackmann, E. & Trauble, H. (1972) *J. Amer. Chem. Soc.* **94**, 4482-4491.
11. Scandella, C. J., Devaux, P. & McConnell, H. M. (1972) *Proc. Nat. Acad. Sci. USA* **69**, 2056-2060.
12. Devaux, P. & McConnell, H. M. (1973) *Ann. N.Y. Acad. Sci.*, in press.
13. Shimshick, E. J. & McConnell, H. M. (1973) *Biochemistry* **12**, 2351-2360.
14. Shimshick, E. J. & McConnell, H. M. (1973) *Biochem. Biophys. Res. Commun.*, in press.
15. Phillips, M. C., Ladbrooke, B. D. & Chapman, D. (1970) *Biochim. Biophys. Acta* **196**, 35-44.
16. Ververgaert, P. H. J., Elbers, P. F., Luitingh, A. J. & van den Berg, H. J. (1972) *Cytobiology* **6**, 86-96.
17. Shimshick, E. J., Kleemann, W., Hubbell, W. L. & McConnell, H. M. (1973) *J. Supramolecular Struct.*, in press.
18. Verkleij, A. J., Ververgaert, H. J., van Deenen, L. L. M. & Elbers, P. F. (1973) *Biochim. Biophys. Acta* **288**, 326-332.
19. Branton, D., Elgsaeter, A. & James R. (1972) in *Mitochondria and Biomembranes* (North-Holland Publisher Co., Amsterdam), pp. 165-172 (Proceedings of the Eighth Meeting of the FEBS, Amsterdam, August 1972).
20. James, R., Branton, D., Wisniewski, B. & Keith, A. (1972) *J. Supramolecular Struct.* **1**, 38-49.
21. Epstein, W. & Fox, C. F. (1970) *J. Bacteriol.* **103**, 273-274.
22. Fox, C. F., Law, J. H., Tsukagoshi, N. & Wilson, G. (1970) *Proc. Nat. Acad. Sci. USA* **67**, 598-605.
23. Davis, B. D. & Mingioli, E. S. (1950) *J. Bacteriol.* **60**, 17-28.
24. Adelberg, E. A., Mandell, M. & Chen, G. C. C. (1965) *Biochem. Biophys. Res. Commun.* **18**, 788-795.
25. Patterson, M. S. & Green, R. C. (1965) *Anal. Chem.* **37**, 854-857.
26. Tsukagoshi, N. & Fox, C. F. (1971) *Biochemistry* **10**, 3309-3313.
27. Slater, E. C. & Bonner, W. D. (1952) *Biochem. J.* **52**, 185-196.
28. Scandella, C. J. & Kornberg, A. (1971) *Biochemistry* **10**, 4447-4456.
29. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randell, R. J. (1951) *J. Biol. Chem.* **193**, 265-275.
30. Hsu, C. C. & Fox, C. F. (1970) *J. Bacteriol.* **103**, 410-416.
31. Linden, C., Keith, A. D. & Fox, C. F. (1973) *J. Supramolecular Struct.*, in press.
32. Overath, P., Schairer, H. U., Hill, F. F. & Lamnek-Hirsch, I. (1971) in *The Dynamic Structure of Cell Membranes*, eds. Wallach, D. F. H. & Fischer, H. (Springer-Verlag, New York), pp. 149-164.
33. Engleman, D. M. (1970) *J. Mol. Biol.* **47**, 115-117.
34. Rice, O. K. (1967) *Statistical Mechanics, Thermodynamics and Kinetics* (Freeman Publishing Co., San Francisco), pp. 354-355.
35. Tsukagoshi, N. & Fox, C. F. (1973) *Biochemistry*, in press.
36. Mavis, R. D. & Vagelos, P. R. (1972) *J. Biol. Chem.* **247**, 652-659.
37. Papahadjopoulos, D., Jacobson, K., Niv, S. & Isac, T. (1973) *Biochim. Biophys. Acta Biomembranes*, in press.
38. Wu, S. H. W. & McConnell, H. M. (1973) *Biochem. Biophys. Res. Commun.*, in press.