Van Handel, E., and Zilversmit, D. B. (1967), *J. Lab. Clin. Med. 50*, 152.
Vesterberg, O., and Svensson, H. (1966), *Acta Chem. Scand. 20*, 820.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Zanetta, J. P., Vincendon, G., Mandel, P., and Gombos, G. (1970), *J. Chromatogr. 51*, 441.

Dynamics of Pyrene Fluorescence in *Escherichia coli* Membrane Vesicles†

S. Cheng, J. K. Thomas, and C. F. Kulpa

ABSTRACT: Pyrene was incorporated into both inner and outer membrane vesicles of *Escherichia coli* and excited by means of a 10-nsec pulse of ultraviolet light from a Q-switched ruby laser. The decay time of the excited state is subsequently measured by monitoring the fluorescence at 400 nm. The half-lives of the pyrene excited states are 85 and 125 nsec at 25° in the inner and outer membrane vesicles, respectively. Substantial differences in behavior between the inner and outer membranes are also illustrated by the temperature dependence of both the lifetime of the pyrene excited state and the absolute yield of pyrene fluorescence. Two structural transitions were observed for the outer membrane vesicles over the temperature range 0–35°. However, the inner membrane vesicles do not show phase transitions over the same temperature range. The rate of decay of the pyrene excited state increases in the presence of added quenchers such as O_2, iodide, and CH_3NO_2 which dissolve primarily in the water phase. The quenching experiments show that the inner membrane vesicles are more permeable than the outer membrane vesicles to these probes. Fluorescence polarization measurements of the microviscosity of the inner and outer vesicles give data which are in substantial agreement with the laser experiments.

Lipid dispersions have been extensively used as model systems to gain an insight into the functions of membranes, and in particular, specialized techniques have been developed to ascertain the conformational state of the membrane components (Cogan *et al.*, 1973; Hubbell and McConnell, 1968; Ladbrooke and Chapman, 1969). The technique of fluorescence polarization has been particularly useful in measuring the fluidity of the membrane systems (Cogan *et al.*, 1973), and recently, an application of the fluorescent probe technique has proved successful in analyzing several features of the factors controlling the permeability of simple micelles (Wallace and Thomas, 1973; Grätzel and Thomas, 1973) and phospholipid dispersions (Cheng and Thomas, 1974). In this latter technique the fluorescent probe such as pyrene is dissolved in the micelle or membrane and excited by means of a pulsed laser. The decay rate of the excited state is subsequently measured either by rapid absorption or emission spectroscopy. The aqueous phase of the system contains a suitable quencher which may penetrate the membrane and react with the excited state of the probe molecules. The rate of the quenching reaction or decay time of the excited state may be used as a measure of the ease of entry of the quenchers into the micelle or membrane and it is possible to discuss the factors which affect the movement of the quencher into the micelle.

In this paper, we report the application of the pulsed laser technique to *Escherichia coli* membrane systems. The microviscosity, the existence of phase transitions, and the structural organization in the hydrocarbon region of phospholipids have been investigated.

Experimental Section

Materials

Media for culture growth was prepared as follows: proteose peptone No. 3 (Difco) 1.0%, beef extract (Difco) 0.1%, and NaCl 0.5%. Galactose was obtained from General Biochemicals Inc. Na_2EDTA was from J. T. Baker Co. Lysozyme was three-times recrystallized from Calbiochem. Sucrose was obtained from Matheson Scientific.

Pure grade pyrene from Fluka was further purified by passage through a silica gel column in cyclohexane and then crystallized from the solvent. 2-Methylanthracene (Aldrich) was crystallized from ethanol. Laboratory distilled water from a Barnsted Still was redistilled from $KMnO_4$. The criterion of purity of the distilled water has been described previously (Cheng and Thomas, 1974), impurity levels are below 10^{-5} mol/l.

Methods

Bacterial Growth and Membrane Preparation. A mutant of *Escherichia coli* 0111:B_4, designated J-5, was used for all experiments. This strain lacks UDP-galactose 4-epimerase and therefore cannot use galactose as a carbon source (Elbein and Heath, 1965). When galactose is added to the medium, it is used solely for the synthesis of lipopolysaccharide. Thus, in the absence of galactose an incomplete lipopolysaccharide is synthesized which lacks a large portion of carbohydrate (Elbein and Heath, 1965; Levy and Leive, 1968). Cells were routinely grown in the presence of 2.5 mM galactose at 37° unless stated otherwise.

Inner and outer membrane vesicles were prepared from spheroplasts by osmotic lysis and isopycnic sucrose gradients

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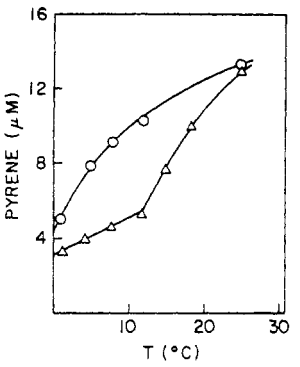

FIGURE 1: Temperature dependence of the incorporation of pyrene into *E. coli* membrane vesicles. The incubation time was 15 min: (○) inner membrane vesicles; (△) outer membrane vesicles.

(Osborn *et al.*, 1972). Gradient fractions containing either inner or outer membranes were pooled and this constituted the working membrane preparations. When outer and inner membranes were isolated from cells grown in the absence of galactose, the sucrose gradients were modified as previously described (Kulpa and Leive, 1972) with the exception that the gradients were centrifuged for 24 hr.

Incorporation of the Fluorescent Probe into Membrane Vesicles. The probes were incorporated into the *E. coli* vesicles by incubating the finely divided probes and vesicles at 25° with gentle stirring for 30 min.

The temperature dependence of the incorporation of the probe into the vesicles was carried out with a series of samples all containing a standard amount of pyrene and vesicles. The mixtures were then incubated for the same period of time at various temperatures. The final concentration of pyrene in the vesicles was measured spectrophotometrically on a Cary 15 spectrophotometer at 337 nm where the extinction coefficient of pyrene, ϵ_M is 54,000 M^{-1} cm^2.

Half-Life Measurements of the Pyrene Singlet Excited State. The rate of decay of the pyrene excited singlet state was followed by fast kinetic spectroscopy which has been described (Grätzel and Thomas, 1973; Cheng and Thomas, 1974). With this technique, lifetimes down to a few nanoseconds could be conveniently measured. The pyrene was excited in the various systems by using the 347.1-nm line from a frequency doubled ruby laser, manufactured by Korad, laser No. K1QP. The output ultraviolet line was about 0.2 J in a pulse of 10-nsec duration. A weak excimer emission was also observed at 490 nm. The excimer appears abruptly with the laser pulse, and corresponds to two adjacent pyrene molecules in the membrane. Only the monomer fluorescence is discussed in this paper.

Fluorescence Polarization Measurements and Evaluation of Microviscosity. The polarization studies were carried out on an Aminco-Bowman spectrophotofluorometer, with polarcoat polarizing filters in both the excitation and detection channels. Since the scattering of the polarized exciting light results in an increase of the measured polarization, interference, and cutoff filters were also placed in the excitation and emission channels to eliminate light from second-order diffraction and scattering from the cell. The treatment of the data and the evaluation of the microviscosity were carried out as previously described (Grätzel and Thomas, 1973; Cheng and Thomas, 1974).

Results

The effect of temperature on the incorporation of pyrene into *E. coli* membrane vesicles is shown in Figure 1. The incubation time was 15 min in each experiment. For the outer membrane vesicles a slow increment of incorporation is observed in the temperature range between 0 and 10° followed by a rapid increase of pyrene uptake from 10 to 20°. For the inner membrane vesicles, the slow uptake of pyrene at lower temperatures is not observed. The insert a and b in Figure 2 show the oscillographic traces of the decay of the pyrene singlet excited state monitored at 400 nm in the outer and inner membrane vesicles, respectively, while Figure 2 itself shows the first-order decay of the excited state. The half-lives of the decay of the pyrene fluorescence are 85 and 125 nsec at 25° for the inner and outer membrane vesicles, respectively. The effect of temperature on the pyrene singlet excited-state half-life is shown in Figure 3. A very sharp decrease in the lifetime of pyrene excited state was observed in the outer membrane vesicles between the temperature range of 0–20° and 25–30°, while a slightly slower decrease was observed between 20 and 25°. These observations are very reproducible. The inner membrane vesicles again show smaller effects of temperature on the pyrene singlet excited-state lifetime between 0 and 30°. These results are also consistent with the studies of the temperature dependence of the absolute yield of pyrene fluorescence measured on an Aminco-Bowman spectrofluorimeter and shown in Figure 4. The fluorescence intensity increases about twofold as the temperature changes from 25 to 5° for the outer membrane vesicles, while a smaller effect was observed for the inner membrane over the same temperature range.

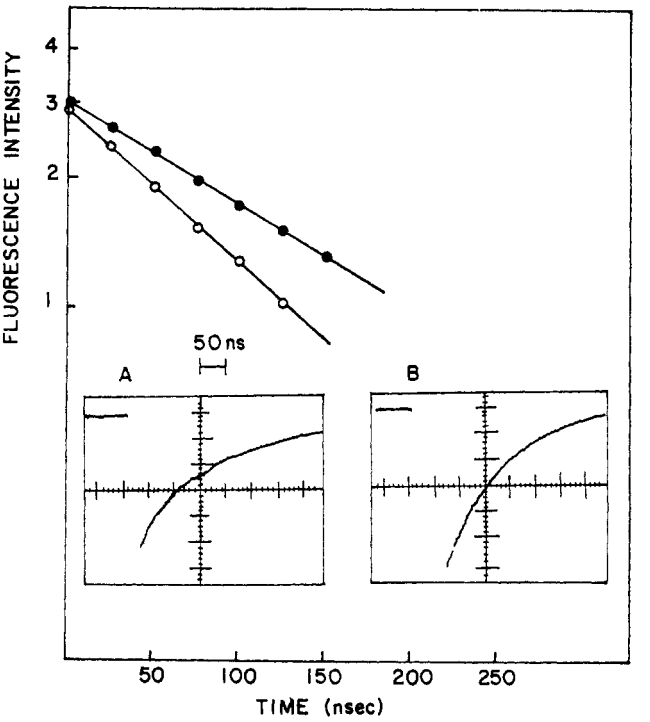

FIGURE 2: Oscillographic trace of the decay of the pyrene singlet excited state. Time zero corresponds to the initial observation of the fluorescence trace. The emission was monitored at 400 nm, 25°. The pyrene concentration is 1×10^{-5} M: (○) inner membrane vesicles; (●) outer membrane vesicles. Inset A: outer membrane vesicles; B: inner membrane vesicles.

The observed rate constant for the first-order decay of pyrene fluorescence in an aqueous solution of micelles containing solubilized pyrene and a quencher Q at much higher concentration than that of the excited pyrene is given by

$$k = \frac{ln\,2}{\tau} + k_2[Q]$$

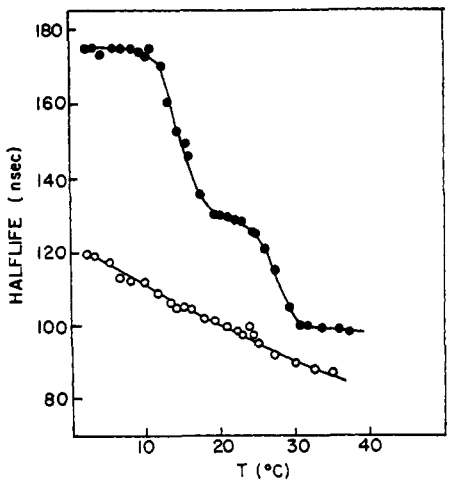

FIGURE 3: Temperature effect on the lifetime of the pyrene excited singlet state: (○) inner membrane vesicles; (●) outer membrane vesicles. Pyrene concentration is 1×10^{6} M.

where τ is the fluorescence half-life of pyrene in the absence of quenchers and k_2 is the second-order rate constant for the quenching reaction.

Figure 5 shows the dependence of the quenching of the pyrene excited state on the nitromethane concentration. From the rates of decay of the pyrene fluorescence and the preceding equation, the second-order rate constants were calculated to be 6.65×10^{8} and 1.15×10^{8} M^{-1} sec^{-1} for the inner and outer membrane vesicles, respectively. Other solutes such as I^- and O_2 also quench the pyrene excited state and the appropriate rate constants are given in Table I. Higher quenching rates are always observed with inner membrane vesicles. The quencher molecules are specifically chosen so that they are predominantly soluble in water, and not in the membrane system. This is essential if the above equation is to be used with [Q] as the bulk concentration. If the quencher has significant solubility in the membrane, then simple pseudo-first-order kinetics are not observed.

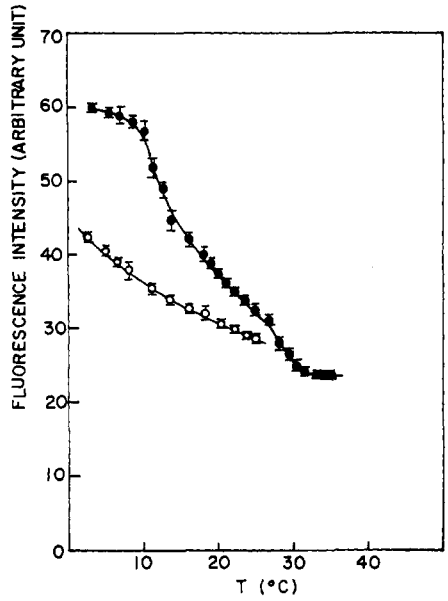

FIGURE 4: Temperature dependence of pyrene fluorescence intensity: (○) inner membrane vesicles; (●) outer membrane vesicles. Pyrene concentration is 1×10^{5} M.

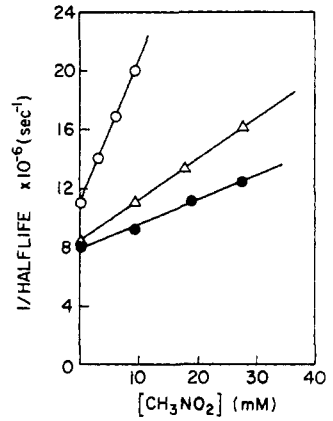

FIGURE 5: Quenching rate constant of CH_3NO_2 with pyrene excited singlet state as a function of quencher concentration: (○) inner membrane vesicles; (●) outer membrane vesicles; (△) outer membrane vesicles in the presence of 10 mM benzyl alcohol.

Benzyl alcohol, which has the skeletal structure of many anesthetics, increases the permeability of membrane vesicles to quencher molecules (Elworthy *et al.*, 1968). The effect of benzyl alcohol on the quenching rate of CH_3NO_2 is shown in Figure 5. In the presence of 10 mM benzyl alcohol, the quenching rate increases about twofold for the outer membrane vesicles. Similar results were observed with iodide quenching (Figure 6).

The pyrene fluorescence lifetime measurements and the quenching rate of the pyrene singlet excited state with added solutes reflect the permeability or rigidity of the host membrane vesicles. The rigidity of the vesicle is also reflected in the microviscosity of the interior of the vesicle. This was evaluated from fluorescence polarization measurement, using 2-methylanthracene as a probe. The degree of polarization of the fluorescence can be related to the viscosity of the medium in which the probe is dispersed by the Perrin equation (Perrin, 1936)

$$\frac{\frac{1}{p} - \frac{1}{3}}{\frac{1}{P_0} - \frac{1}{3}} = 1 + \frac{KT\tau}{\eta V_0}$$

TABLE I: Reaction Rates, k (M^{-1} sec^{-1}), for the Quenching of Pyrene Fluorescence in *E. coli* Membrane Vesicles.[a]

Quencher	*E. coli* (+Galactose)[b]		*E. coli* (−Galactose)	
	IM[c]	OM	IM	OM
O_2	5.65×10^{9}	3.34×10^{9}	5.2×10^{9}	4.5×10^{9}
I^-	2.54×10^{8}	3.44×10^{6}	2.8×10^{8}	8.8×10^{7}
CH_3NO_2	6.65×10^{8}	1.15×10^{8}	6.5×10^{8}	3.38×10^{8}

[a] The reactions were carried out at 25°. [b] +Galactose or −galactose refers to the presence or absence of galactose in the growth medium. [c] IM refers to inner membrane and OM refers to outer membrane.

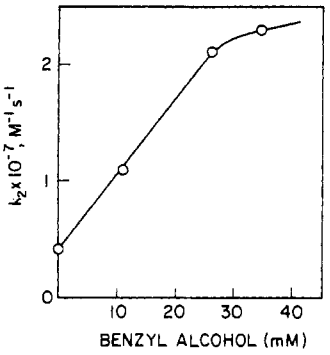

FIGURE 6: Effect of benzyl alcohol on the I^- quenching rate with pyrene excited singlet states in the outer membrane vesicles.

where P_0 is the degree of polarization measured in an extremely viscous medium, τ is the average lifetime of the probe molecules in the excited state, V_0 is its effective volume, and η is the viscosity. In hydrocarbon solvents, the fluorescence decay time of 2-methylanthracene is 3.5 nsec and an effective volume of 105 $Å^3$ (Lyklema and Overbeck, 1961) may be taken to calculate the microviscosity. The microviscosities are 37 and 73.5 cP at 25° for inner and outer membrane vesicles, respectively, again illustrating the less rigid structure of the inner membrane.

The membrane vesicles which are isolated from cells grown in a medium lacking galactose show thermal phase transitions similar to those of inner and outer membrane vesicles isolated from the cells grown in the presence of galactose. However, in the quenching studies a remarkable difference is observed between outer membrane vesicles from cells grown with or without galactose. This effect is illustrated in Table I. The quenching rate of the pyrene fluorescence by I^- increases about 25-fold in the membrane vesicles isolated from cells which are grown in a medium containing no galactose. These outer membrane vesicles contain fewer carbohydrates because their lipopolysaccharide is incomplete (Elbein and Heath, 1965; Levy and Leive, 1968). Lipopolysaccharide is known to reside in the outer membrane of gram-negative bacteria, such as *E. coli* or *Salmonella* (Osborn *et al.*, 1972).

Discussion

Fluorescent probes have been used previously to study microviscosities of cell membranes (Cogan *et al.*, 1973). Pyrene, with its longer lived excited singlet state, offers several advantages over the earlier probes used, and a wider time scale of events can be studied inside the membrane vesicles (Wallace and Thomas, 1973; Grätzel and Thomas, 1973; Cheng and Thomas, 1974). In particular, the movement of the excited state from the interior of the membrane to the surface can be studied. In earlier work on micellar systems, a reaction of excited molecules with amino compounds to form excited complexes has been observed (Grätzel and Thomas, 1973). Similar effects are suggested in the present systems. Amino and quaternary ammonium groups at the surface of the membrane may rapidly quench the excited state, with the decreased lifetime being indicative of the transit time of the probe from the interior to the surface. The transit time is many tens of nanoseconds, however, and conventional probes (2-methylanthracene) with excited-state lifetimes much less than 10 nsec do not live to react with the surface groups. The normal lifetime of pyrene is many hundreds of nanoseconds however, and this makes it an ideal probe for the above mentioned dynamic process.

The effect of temperature (Figure 1) on the incorporation of pyrene into *E. coli* membrane vesicles indicate the dissimilarity between inner and outer membrane visicles. The incorporation of pyrene into the outer membrane vesicles is relatively slow from 0 to approximately 10°, while above 10° there is a marked increase of pyrene uptake. Such a drastic change of pyrene uptake by the membrane was not observed with the inner membrane vesicles. Differences between the inner and outer membranes are also illustrated by the effect of temperature on the lifetime and fluorescence intensity of the pyrene excited singlet state in these membrane vesicles (Figures 3 and 4). The lifetime of the pyrene excited state in *E. coli* membrane vesicles is much shorter than that in hydrocarbon solvents where a value of ~400 nsec is observed (Birks, 1970). Shorter lifetimes for the pyrene excited states have been observed in cetyltrimethylammonium bromide micelles where the surface bromide ions quench the excited state (Grätzel and Thomas, 1973; Patterson and Vieil, 1973). Since about 70% of the phospholipid in *E. coli* J-5 is phosphatidylethanolamine (White *et al.*, 1972) and it is established that amines quench excited states (Grätzel and Thomas, 1973), then the shortened fluorescence lifetimes of pyrene singlet excited state are attributed to a quenching effect of the amino group of phosphatidylethanolamine in the vesicles. The temperature dependence of both the lifetimes and fluorescence intensity measurements clearly indicate that a phase transition is observed with the outer membrane vesicles but not with the inner membrane vesicles.

Identical results have been observed with at least four different preparations of *E. coli* membrane vesicles, and two structural transitions are always detected for the outer membrane. The first transition occurs between 10 and 20° followed by a less-pronounced change between 20 and 25° and a second transition occurs between 25 and 30°. At temperatures above 30° a plateau is reached and no further phase transitions are observed. Phase transitions and the temperature characteristic of *E. coli* membrane vesicles have been studied by physical techniques (Esfahani *et al.*, 1971; Haest *et al.*, 1969; Schairer and Overath, 1969) and transport studies (Kaback, 1970, 1972; Shechter *et al.*, 1972). In particular, X-ray diffraction and fluorescence studies applied to membrane vesicles into which dansylphosphatidylethanolamine has been incorporated, show two phase transitions with temperature (Shechter *et al.*, 1972). However, our studies do not show a phase transition for the cytoplasmic membrane.

The present results have been interpreted in terms of lipid phase transitions that are correlated with changes in membrane fluidity. The transition that occurs between 10 and 20° is suggested to coincide with the melting of paraffin chains of the membrane phospholipids.

The lifetime measurement of the pyrene excited singlet state gives some measure of the fluidity of the paraffin of the membrane phospholipid (Cheng and Thomas, 1974), the fluorescence lifetime reflecting the movement of pyrene inside the vesicles to the surface. The data indicate that the outer membrane vesicles are more rigid than the inner membrane vesicles. This was also confirmed by the fluorescence polarization studies which gave microviscosities of 37 and 73 cP for the inner and outer membrane vesicles, respectively.

Although the pyrene excited singlet state is partially quenched by the amine group of the phospholipids, it can be further quenched by molecules such as CH_3NO_2, I^-, and O_2 which penetrate into the membrane. In Table I it can be seen that the quenching rate constants of various quenchers with pyrene are higher in the inner membrane than those in the outer membrane vesicles.

All the data show that the inner membrane vesicles are less rigid than the outer membrane vesicles. The addition of the anesthetic, benzyl alcohol, increases the permeability of the outer membrane vesicles. It has been suggested that this type of molecule is absorbed at the membrane surface and tends to push the head groups on the membrane surface further apart (Elworthy *et al.*, 1968). A carbohydrate-deficient lipopolysaccharide in the outer membrane also increases the penetration of quenchers into the vesicles (Table I). However, the phase transitions in these vesicles are identical with those observed in the vesicles with the normal compliment of carbohydrate. The remarkable difference in I^- quenching rate between outer membrane vesicles from cells grown with galactose and those from cells grown without galactose can be explained in terms of the repulsion between I^- and the negative charges of the hydroxyl group of the polysaccharide of lipopolysaccharide. The more rigid nature of the complete polysaccharide obtained when cells are grown in galactose will also inhibit the movement of I^- into the membrane.

The possible functional importance of thermatropic phase transitions in lipid of artificial and biological membranes has been discussed by several authors (Cogan *et al.*, 1973; Hubbell and McConnell, 1968; Kaback, 1972). It has been shown, that these transitions are determined by several factors: (a) the length and the degree of saturation of the hydrocarbon chain (Ladbrooke and Chapman, 1969); (b) the electrostatic interactions of the polar head groups of the membrane lipids; and (c) the presence of bivalent cations, cholesterol, peptides, or protein (Träuble, 1971; Chapman and Urbina, 1971; Long *et al.*, 1971). Such phase transitions within the lipid core of membranes when affected by the above mentioned biochemical or biophysical conditions might have physiological functions in the regulation of membrane permeability and kinetic properties of membrane-associated enzymes of thermosensitive bacteria, plants, and animals (Raison *et al.*, 1971). The detailed structure of lipid and protein in the biological membrane is still uncertain. Further studies of the interaction between membrane proteins and membrane lipids in *E. coli* vesicles is in progress.

References

Birks, J. B. (1970), Photolysis of Aromatic Molecules, New York, N. Y., Wiley-Interscience.

Chapman, D., and Urbina, J. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 12*, 169–173.

Cheng, S., and Thomas, J. K. (1974), *Biochemistry*, to be submitted for publication.

Cogan, J., Shinitzky, M., Weber, G., and Nishida, T. (1973), *Biochemistry 12*, 521–528.

Elbein, A. D., and Heath, E. C. (1965), *J. Biol. Chem. 240*, 1919–1925.

Elworthy, P. H., Florence, A. T., and MacFarlane, C. B. (1968), Solubilization by Surface Active Agents, London, Chapmann and Hall.

Esfahani, M., Limbrick, A. R., Knutton, S., Oka, T., and Wakil, S. J. (1971), *Proc. Nat. Acad. Sci. U. S. 68*, 3180–3184.

Grätzel, M., and Thomas, J. K. (1973), *J. Amer. Chem. Soc. 95*, 6885.

Haest, C. W. M., DeGier, J., and Van Deenen, L. L. M. (1969), *Chem. Phys. Lipids 3*, 413–417.

Hubbell, W. L., and McConnell, H. M. (1968), *J. Amer. Chem. Soc. 93*, 314–326.

Kaback, H. R. (1970), *Annu. Rev. Biochem. 39*, 561.

Kaback, H. R. (1972), *Biochim. Biophys. Acta 265*, 367.

Kulpa, C. F., and Leive, L. (1972), *in* Membrane Research: First ICN–UCLA Symposium on Molecular Biology, Fox, C. F., Ed., New York, N. Y., Academic.

Ladbrooke, B. D., and Chapman, D. (1969), *Chem. Phys. Lipids 3*, 304–355.

Levy, S. B., and Leive, L. (1968), *Proc. Nat. Acad. Sci. U. S. 61*, 1435–1439.

Long, R. A., Hruska, F. E., Gesser, H. D., and Hsia, J. C. (1971), *Biochem. Biophys. Res. Commun. 45*, 167–173.

Lyklema, J., and Overbeck, J. Th. G. (1961), *J. Colloid Sci. 16*, 501.

Osborn, M. J., Gander, J. E., Parisi, E., and Carson, J. (1972), *J. Biol. Chem. 247*, 3962–3972.

Patterson, L. K., and Vieil, E. (1973), *J. Phys. Chem. 77*, 1191–1192.

Perrin, F. (1936), *J. Phys. Radium 7*, 1–10.

Raison, J. K., Lyons, J. M., Mehlhorn, R. J., and Keith, A. D. (1971), *J. Biol. Chem. 246*, 4036–4040.

Schairer, H. U., and Overath, P. (1969), *J. Mol. Biol. 44*, 209–214.

Shechter, E., Gulik-Krzywicki, T., and Kaback, H. R. (1972), *Biochim. Biophys. Acta 274*, 466–477.

Träuble, H. (1971), *Naturwissenschaften 58*, 277–284.

Wallace, S. C., and Thomas, J. K. (1973), *Radiat. Res. 54*, 49–62.

White, D. A., Lennarz, W. J., and Schnaitman, C. A. (1972), *J. Bacteriol. 109*, 686–690.