Structure of the Lipid Phase in Cell Envelope Vesicles from *Halobacterium cutirubrum*†

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ABSTRACT: The flexibility of the lipids in *Halobacterium cutirubrum* cell envelope vesicles and extracted lipid dispersions was investigated with the spin-label method using fatty acid derivatives of the general formula $CH_3(CH_2)_m$-$CR(CH_2)_nCOOH$ (R = *N*-oxyl-4′,4′-dimethyloxazolidine) as probes. The cell envelope membranes were found to differ from isolated lipids in that (1) the polarity around the spin-label group of labels where $[m,n]$ = [12,3], [7,6], and [5,10] is considerably higher in membranes than in lipids; (2) the order parameter, S_n, in membranes is unusually high and nearly constant for $n < 14$, while in lipids the usual flexibility gradient was observed for this region; and (3) thermal transitions for labels [12,3] and [5,10] occurring in lipid dispersions, as evidenced by the temperature dependence of S or of the motion parameter, τ, are absent in membranes. For label [1,14], however, the environment around the nitroxyl group is apparently similar in both membranes and extracted lipids and a thermal transition occurs at the same temperature in both systems. These observations suggest that in *H. cutirubrum* cell envelopes the membrane proteins cause all but a narrow center portion of the lipid bilayer to become more ordered. Since the estimated lipid content of these membranes (lipid: protein ratio <0.2) is relatively low, it is possible that such immobilization is due to the existence of a protein matrix in the membrane.

The extremely halophilic bacterium *Halobacterium cutirubrum* requires sodium chloride concentrations of at least 3 M for growth and is readily lysed when the salt concentration is lowered to less than 1 M (Larsen, 1967). Its cell envelope consists mostly of lipoproteins and contains more than 90% of the cellular phosphorus. The major phospholipid was shown to be a phosphatidyl glycerophosphate containing two ether-linked long phytanyl side chains (Kates *et al.*, 1965). The remaining lipids and even the main carotenoids (C_{50}-carotenes) are phytyl derivatives (Kushawa *et al.*, 1972); therefore, the hydrocarbon part of the membrane seems to be rather uniform.

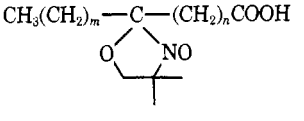
The bonds that hold the membrane together are nonionic; it is most likely that hydrophobic interactions are involved (Brown, 1965; Lanyi, 1971), but some hydrogen bonding between membrane proteins also may be important (Lanyi, 1971).

These halophilic bacteria have the remarkable ability to accumulate potassium ions against a large concentration gradient (Christian and Waltho, 1962). The intracellular potassium, at a concentration of almost 3 M, was found not to be bound to an intracellular matrix (Lanyi and Silverman, 1972). The absence of a rigid cell wall, together with the remarkable physicochemical and metabolic properties of the cell envelope, make this organism a valuable tool for investigations of structure–function relationships in membranes. In the present work, we have investigated the physical state of the lipid phase in the cell envelope of *H. cutirubrum* by incorporating spin-labeled fatty acids into membrane vesicles. The electron paramagnetic resonance spectra of such labels can yield valuable information on the structure of membranes (McConnell and McFarland, 1970; Hubbell and McConnell, 1971; Seelig and Hasselbach, 1971; Henry and Keith, 1971). Spin-label studies on whole cells of *Halobacterium salinarium* were reported earlier by Hsia *et al.* (1971).

Material and Methods

H. cutirubrum cells were grown and harvested as described by Hochstein and Dalton (1968). Membrane vesicles were prepared by freeze-thawing pellets of sedimented whole cells. The lysed cell pastes were resuspended in a buffer solution containing 0.05 M Hepes[1] (pH 7), 3.4 M NaCl, and "basal salts" (which refers to salts other than NaCl (Lanyi, 1971)), homogenized briefly in a Sorvall Omnimixer, centrifuged for 20 min at 35,000*g*, and finally resuspended in the same buffer medium (25 mg of protein/ml). These vesicles still exhibit considerable respiration (J. K. Lanyi, unpublished). Protein concentrations of vesicle preparations were determined by the biuret method, in the presence of 1% deoxycholate. The total lipids were extracted by shaking cells with chloroform–methanol (3:1). After five extractions the organic phases were combined and evaporated to dryness *in vacuo*. The lipids were redissolved in chloroform and were determined gravimetrically. To prevent isomerization of the carotenoids (Kushawa *et al.*, 1972), the lipids were stored in darkness at −15° until used. Vesicles, stripped of outer envelopes, were obtained from complete cell envelopes by short incubation in 0.05 M Hepes (pH 7) containing 20 mM $MgCl_2$ and then centrifugation (Marshall *et al.*, 1969; W. Stoeckenius, personal communication). Under these conditions, 20% of the total envelope protein was lost, as compared to a value of 15% reported (Marshall *et al.*, 1969).

For the present work, fatty acid spin labels of the general formula



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[1] Abbreviation used is: Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

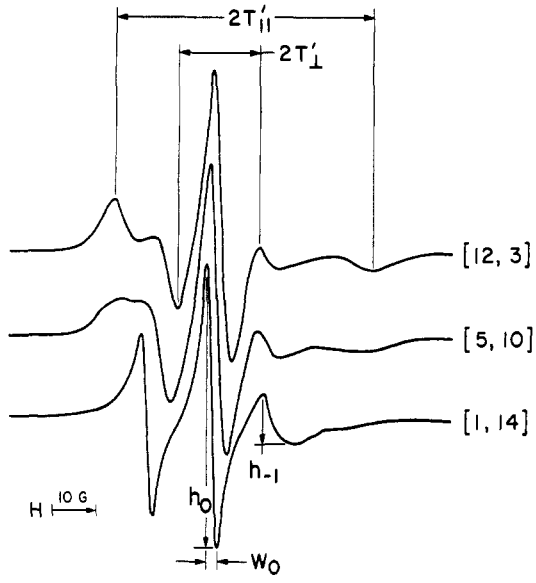

FIGURE 1: Electron paramagnetic resonance spectra of fatty acid spin-label [*m*,*n*] in cell membrane vesicles from *H. cutirubrum* at 37°. Instrument settings: microwave power, 1 mW; modulation amplitude, 0.9 G; average of 64 scans.

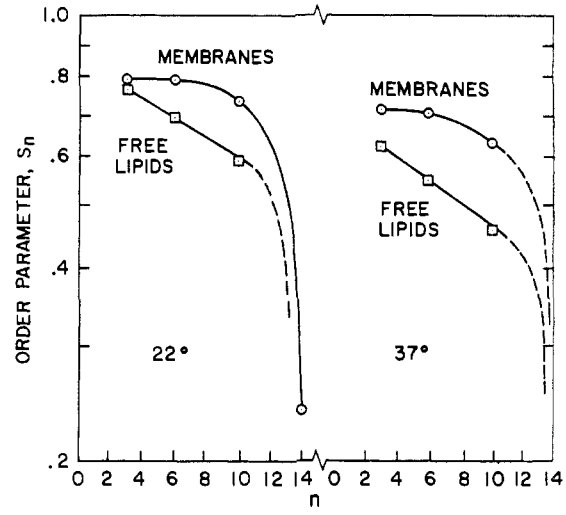

FIGURE 2: The order parameter S_n as a function of n for spin-label [*m*,*n*] in cell membrane vesicles and lipid extracts of *H. cutirubrum* at 22 and 37°. Vesicles and lipids are suspended in 3.4 M NaCl, 0.05 M Hepes (pH 7.0), and "basal salts."

were used. The labels where [*m*,*n*] = [12,3], [5,10], and [1,14] were obtained commercially (SYVA, Palo Alto, Calif.), and the label [7,6] was kindly provided by Dr. B. G. McFarland. The spin labels were incorporated into *H. cutirubrum* vesicles following a procedure of Seelig and Hasselbach (1971). The concentration of the vesicle suspension was usually 25 mg of protein/ml; the amount of added label was 25 μg or less, resulting in a ratio of lipid to labeled fatty acid of at least 200:1. The extracted lipids were labeled similarly. Here, the labels were added to the chloroform–lipid solution and the solvent was evaporated in a 10-ml volumetric flask. The residue was dispersed first in water, by agitating on a Vortex mixer, then the basal salts (the appropriate amount of a concentrated stock solution) and the NaCl (in solid form) were added, and the shaking was repeated.

All electron paramagnetic resonance (epr) spectra were recorded on a Varian V-4502 X-band spectrometer in connection with a signal-averaging system (Nicolet, Model 1072). The samples contained in 50-μl capillaries were mounted in a special holder (design of R. D. Kornberg, described in McFarland, 1973) in the Varian variable-temperature accessory. Attached to the capillary was a thermocouple made from very thin wires (0.01-mm diameter; Omega Eng., Stamford, Conn.). Thus, with a suitable meter (Thermocouple Readout Meter, Model T-5; Omega Eng., Stamford, Conn.) it was possible to accurately monitor the temperature at the center of the cavity without introducing much noise. An incident microwave intensity of 1–3 mW and a modulation amplitude of 0.9 G was used throughout the work.

Two different methods were used to interpret the recorded epr spectra. The fatty acid spin labels undergo anisotropic motion around their long molecular axis (Libertini *et al.*, 1969). To measure this motion quantitatively, Seelig (1970) introduced an order parameter, S, that can be calculated from the experimental data with

$$S = \frac{T'_{||} - T'_{\perp}}{T_{zz} - 0.5(T_{xx} + T_{yy})} \frac{a}{a'} \quad (1)$$

Here $T'_{||}$ and $T'_{\perp}$ correspond to the separation of the outer and inner hyperfine extrema as shown in Figure 1; T_{xx}, T_{yy}, and T_{zz} are obtained from crystal parameters and a'/a is a correction for polarity (Hubbell and McConnell, 1971). Using the values of R. C. McCalley (cited by McFarland, 1973) for T_{xx}, T_{yy}, and T_{zz} (6.31, 5.83, and 31.91 G) and the following equations

$$a = 1/3(T_{xx} + T_{yy} + T_{zz}) \quad (2)$$

$$a' = 1/3(T'_{||} + 2T'_{\perp}) \quad (3)$$

one can rewrite eq 1 and obtain

$$S_n = 0.568\frac{T'_{||} - T'_{\perp}}{a'} \quad (4)$$

(The term n is used to distinguish between different labels and corresponds to n in the general formula for fatty acid spin labels as given above.) It is often found that the experimental value of $T'_{||}$ cannot be obtained easily, especially for label [1,14] (Figure 1). In these cases, meaningful information can still be obtained by calculating an empirical motion parameter, τ (Keith *et al.*, 1970), according to

$$\tau_n = 6.5 \times 10^{-10} W_0 \left[\left(\frac{h_0}{h_{-1}}\right)^{1/2} - 1\right] \quad (5)$$

where W_0 and h_0 are the width and the height of the center line, h_{-1} is the height of the high field line (Figure 1), and n is as defined above.

Results

At room temperature, *H. cutirubrum* vesicles exhibit epr spectra which indicate that the fatty acid labels experience rather strong "immobilization." If the nitroxyl group is attached nearer the methyl end of the hydrocarbon chain, the motional freedom of the group increases. Figure 2 is a semilogarithmic plot of the order parameter S *vs.* the position

TABLE I: Spectral Parameters of Spin Labels Incorporated into Membranes and Lipids of *H. cutirubrum*.

Preparation	Solvent[a]	Label	$T = 22°$ S_n	τ_n ($\times 10^{10}$)	a'	$T = 37°$ S_n	τ_n ($\times 10^{10}$)	a'	Treatment
Membrane vesicles	A	[12,3]	0.79	235	15.4	0.71	87	14.8	None
	B	[12,3]	0.75	183	15.4	0.70	56	14.8	None
	A	[12,3]	0.78	215	15.5	0.71	85	14.8	Heat denaturation
	A	[7,6]	0.79	187	15.2	0.70	68	14.7	None
	B	[7,6]	0.78	157	15.1	0.67	53	14.7	None
	A	[7,6]	0.78	107	15.1	0.67	53	14.7	Heat denaturation
	A	[5,10]	0.73	110	15.0	0.63	57	14.6	None
	B	[5,10]	0.72	101	14.9	0.62	54	14.6	None
	A	[5,10]	0.73	97	15.1	0.62	50	14.8	Heat denaturation
	A	[1,14]	0.24	26	14.1	<0.24	16	13.9	None
	B	[1,14]	<0.24	24	14.1	<0.24	13	13.9	None
	A	[1,14]	0.30	43	14.8	0.27	35	14.6	Heat denaturation
Lipid extracts	A	[12,3]	0.76	106	14.6	0.62	66	14.5	None
	A	[7,6]	0.69	95	14.5	0.55	47	14.4	None
	A	[5,10]	0.59	44	14.3	0.46	27	13.9	None
	A	[1,14]	≪0.24	14	13.8	≪0.24	10	13.8	None

[a] Solvents: (A) 3.4 M NaCl, 0.05 M Hepes, + (B); (B) $MgCl_2 \cdot 6H_2O$, 20 g/l.; KCl, 2 g/l.; $CaCl_2 \cdot 2H_2O$, 0.2 g/l. (= "basal salts").

of the label group along the hydrocarbon chain at two temperatures. We found that S is almost constant for the first ten carbon–carbon bonds and then it decreases sharply, especially at the higher temperature. From the change in S *vs.* n and from the observation that the free-radical group is only slightly reduced by ascorbate, we infer that the fatty acid labels are incorporated into the lipid layer of the membrane in accordance with earlier interpretations (McConnell and McFarland, 1970).

The membrane vesicles are stable for a short while in a solution of basal salts, without NaCl. Therefore, labeled vesicles in high salt buffer were centrifuged, diluted with basal salt solution, sedimented again, and then resuspended in basal salt buffer. Under these conditions, the labels show almost the same behavior as before, with only a minute decrease in order (Table I). Thus, high salt concentrations *per se* do not influence the mobility of the labels in the vesicles. Denaturation of the membrane proteins, by heating the vesicles for 5 min at 70° likewise has only a small effect on the first three labels. Surprisingly, however, the motion of label [1,14] seems somewhat more restricted after this treatment. Table I also contains values for a', the hyperfine splitting constant, which is a monitor for the polarity of the environment around the nitroxyl group. The values of a', calculated according to eq 3, indicate that labels [12,3], [7,6], and [5,10] incorporated into envelope vesicles oscillate in an environment which has a polarity between that of water and ethanol ($a' = 15.6$ and 14.6 G, respectively (Seelig and Hasselbach, 1971)). Only the spin-carrying group of label [1,14] is located in an environment that has an a' value representative of hydrocarbons; *e.g.*, Hubbell and McConnell (1971) found that a' for the free nitroxyl ring dissolved in hexane is 14.1 G.

These results should be contrasted to the data obtained for spin-labeled total lipids from *H. cutirubrum*. It is obvious (Table I) that the spin-label groups incorporated into the total lipids experience a polarity that is representative of a hydrocarbon environment, with the exception of label [12,3], of course, where the radical group is near the polar groups of the lipids. Furthermore, in the lipid dispersion (Figure 2), log S is almost a linear function of n, as predicted by a model in which the freedom of motion of the label depends on isomerization processes around adjacent carbon–carbon bonds along the hydrocarbon chain (Hubbell and McConnell, 1971). The value for S_{14} does not fall onto the line. The reason for this deviation is presently not clear but it is a common observation in other systems (McFarland, 1973). In addition, we found that the observed polarity values, a', do not change appreciably whether the lipids are dispersed in the high salt solution, in basal salts only, or in water. In the latter case, however, the mobility of the fatty acid spin labels increases considerably. This may be caused by the absence of charge shielding effects in water as compared to those at high salt concentration and the concomitant repulsion between the negative charge on the carboxyl group of the fatty acids and the double negatively charged phosphate groups of the major lipids. A more detailed report on *in vitro* studies with total lipids from *H. cutirubrum* will be given elsewhere (A. Esser, in preparation).

The findings of Steim *et al.* (1969) indicate that lipids in membranes can undergo the same temperature-induced transitions as the extracted lipids *in vitro*. For this reason, order and motion parameters were measured at different temperatures. Figures 3, 4, and 5 show the results for membrane vesicles and lipid extracts, both in high salt solutions, obtained with different spin labels. It is evident that for the lipids all three labels show definite transitions, albeit at different temperatures, while in the vesicles a transition is seen only with label [1,14]. This latter observation is not due to the fact that in this case τ was used as the variable parameter instead of S, since one can observe the same transition point for label [12,3] in lipids in both S_3 *vs.* T and τ_3 *vs.* T^{-1} plots, while no transition is seen in similar plots in membranes. Unfortunately, it is not possible to plot S_{14} *vs.* T because no reliable data for $T'_{||}$ can be measured. (The

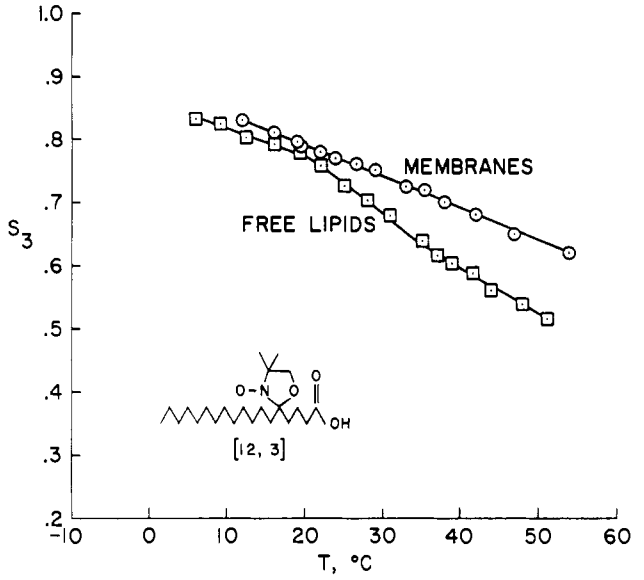

FIGURE 3: Plot of the order parameter S_3 for spin-label [12,3] in membrane vesicles and lipid extracts of *H. cutirubrum* as a function of temperature. Lipid:spin-label ratio of 200:1; medium as in Figure 2.

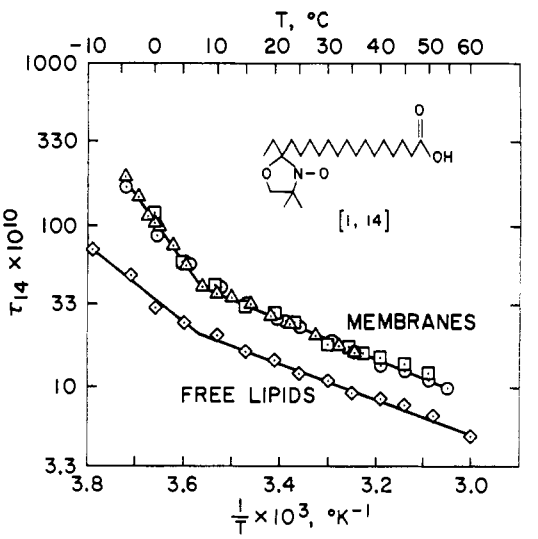

FIGURE 5: Arrhenius plot of τ_{14} for spin-label [1,14] in membrane vesicles and lipid extracts of *H. cutirubrum*. Medium as in Figure 2: (△,◇) lipid:spin-label ratio, 200:1; (□) lipid:spin-label ratio, 100:1; (○) lipid:spin-label ratio, 30:1.

rationale for using Arrhenius plots in the case of τ is given by Raison *et al.* (1971).) Also note that for label [1,14] the transition point occurs at the same temperature in lipids and in membranes (Figure 5). The measurement with vesicles could not be extended to temperatures much higher than 45° due to rapid loss of the free radical. It was found that this reduction could be partially blocked by adding respiration inhibitors. No inhibitors were used during these studies, rather the vesicles were stored for at least three days in the refrigerator to exhaust their intrinsic substrates.

The lipid content of *H. cutirubrum* cell envelope preparations was found to be relatively low. In complete envelope vesicles, the lipid:protein ratio was 0.16 ± 0.02. In membranes devoid of the particles of the outer envelope (Marshall *et al.*, 1969), the value obtained was 0.20 ± 0.02 (seven determinations). No variation in lipid:protein ratio with culture age, such as seen for *H. salinarium* (Brown and Stevenson, 1971), was observed for *H. cutirubrum* membranes. The apparent relative lipid content of membranes is strongly influenced by the amount of cytoplasmic proteins adsorbed during preparation (Dodge *et al.*, 1963) and the values obtained may be low due to this effect. In the presence of NaCl concentrations over 3 M, such as used in the preparation of these vesicles, however, we do not expect that a significant amount of nonspecific charge interaction can be present between the membrane and cytoplasmic proteins.

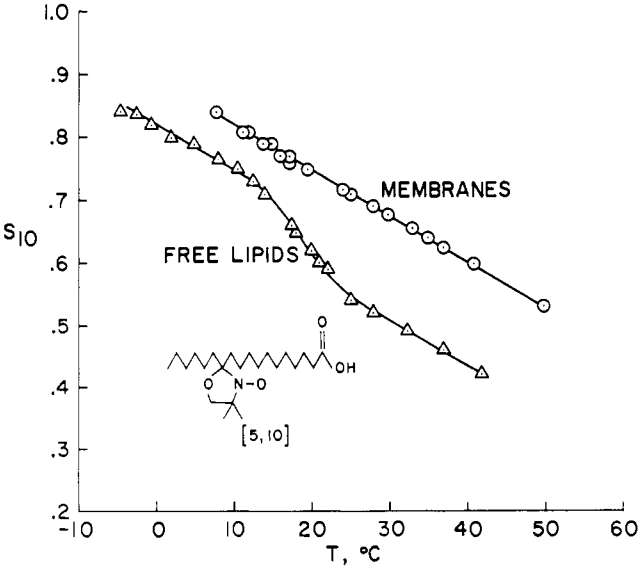

FIGURE 4: Plot of the order parameter S_{10} for spin-label [5,10] in membrane vesicles and lipid extracts of *H. cutirubrum* as a function of temperature. Other conditions as in Figure 3.

Discussion

Several physical methods (for instance X-ray diffraction, thermal analysis, nuclear magnetic resonance, and epr spectroscopy) have indicated that the lipids of membranes are arranged in a bilayer structure (Stoeckenius and Engleman, 1969). It was also shown that highly fluid lipid regions in the membrane can be detected which are comparable to the state of lipids in lipid vesicles (Hubbell and McConnell, 1969; Steim *et al.*, 1969; Davis and Inesi, 1971; Oldfield and Chapman, 1972). Our own data presented here are compatible with the notion that the lipids are arranged in a bilayer. However, in contrast to other results with the spin-label method, it seems that our data give no indication of a *highly* fluid lipid phase in *H. cutirubrum* cell envelope vesicles.

Although the probes used can provide a very sensitive measure of the physical state of their environment, we do not know precisely where the probes are localized in the membrane. While we can be quite certain that the fatty acids are partitioned into the lipid phase of the membrane, we have at the moment no knowledge whether this phase is homogeneous or heterogeneous and, therefore, we do not know on which region the labels will report. However, for reasons discussed explicitly by Oldfield *et al.* (1972), we can assume that the fatty acid spin labels will certainly detect the most fluid regions of a heterogeneous phase. Yet these regions in cell

TABLE II: Order and Motion Parameters for Different Membranes.

Species	Room Temperature S_n	Room Temperature τ_n ($\times 10^{10}$)	Growth or Body Temp S_n	Growth or Body Temp τ_n ($\times 10^{10}$)	Author(s)
H. cutirubrum vesicles	S_3 0.79	τ_3 235	S_2 0.71	τ_3 87	This work
	S_{10} 0.73	τ_{10} 110	S_{10} 0.63	τ_{10} 67	
Rabbit sarcoplasmic vesicles	S_3 0.63				Seelig and Hasselbach (1971)
	S_{10} 0.35				McConnell *et al.* (1972)
Lobster, walking leg nerve fiber	S_3 0.63				Hubbell and McConnell (1971)
	S_{10} 0.45		(= Room temperature)		
Mycoplasma laidlawii (stearate enriched)		τ_{10} ~65		τ_{10} ~45	Tourtellotte *et al.* (1970)
Rat liver mitochondria		τ_{10} ~43		τ_{10} ~40	Raison *et al.* (1971)
Potato mitochondria		τ_{10} ~40		(= Room temperature)	Raison *et al.* (1971)
Fish liver mitochondria		τ_{10} ~41		(= Room temperature)	Raison *et al.* (1971)
Neurospora mitochondria		τ_{10} ~47		τ_{10} ~35	Keith *et al.* (1970)
Yeast mutant SH-1		τ_{10} ~33		τ_{10} ~28	Henry and Keith (1971)

envelope membranes of *H. cutirubrum* appear to have the most highly ordered state and exert the strongest restriction on the motion of the spin-labels reported so far for any biological membrane. Table II is a compilation of several S and τ values for different membranes from available literature data. While more data were found for room temperature experiments, a more valid comparison should be made between S or τ values of membranes at their respective growth or body temperature.

On the other hand, when the same labels are incorporated into aqueous dispersions of lipid extracts from *H. cutirubrum*, spectra are recorded which indicate fast anisotropic motion of the labels. This is not too surprising since practically all lipids of this organism are phytyl derivatives (Kushawa *et al.*, 1972), *i.e.*, contain branched chains and branching is known to increase the fluidity of hydrocarbons. The motion of the spin probes is slightly restricted when the lipids are dispersed in concentrated salt solution (3.4 M NaCl), equivalent to their natural environment. Still, there is a remarkable difference between the freedom of motion of the fatty acids in the saline lipid dispersion and in the membrane preparation (Figure 2). This difference could be due to denser packing of the lipid phase in the membrane, or the motion could be restricted because of interactions between membrane proteins and lipids. The results presented here are presently interpreted to favor the latter conclusion. First, membranes from *H. cutirubrum* contain such low amounts of lipids (lipid: protein ratio <0.2) that probably no large lipid domains can occur. Second, we found that not only was the motion of the labels in the membranes restricted, but the label environment is rather polar (Table I). This polar environment in the membranes is not introduced by the high salt concentration, since the nitroxyl group oscillates in an environment typical for hydrocarbons in the lipid dispersion even in saline solution. Furthermore, while the motion of the spin label increases when the nitroxyl group is placed away from the polar head group (increasing n), in a fashion predicted by the theory, there is almost no change found in membranes at $n < 14$ (Figure 2). This indicates that in the latter case the hydrocarbon chain can be pictured as an almost "stiff" stick, up to the tenth carbon. And, finally, no temperature-induced transitions were detected in the membranes with labels [12,3] and [5,10], such as are found in lipid dispersions. (The results with label [1,14] are discussed below.) Of course, it is not clear whether the observed temperature transitions in the lipid extracts are characteristic for these lipids or whether they are merely induced by the added fatty acids. Henry and Keith (1971) emphasized that the fatty acids may be considered as impurity pools in the lipid phase. At the temperature of the phase transitions, the matrix molecules become less organized and the energy barriers to motion at the impurity boundaries should change. In any case, it is important to note that the transitions—whatever their cause may be—are suppressed in the membrane. Again, this effect can be explained most easily by assuming that the proteins in the membrane restrict any reorganization of the lipid phase.

The behavior of spin-label [1,14] is different and therefore warrants some additional discussion. With this label, the temperature-induced changes can be detected in the membrane as well as in the lipid extracts (Figure 5). Furthermore, the polarity is *similar* in both cases and the transition occurs at the *same* temperature. It is tempting to speculate that perhaps membrane proteins do not penetrate deep enough into the bilayer to influence the label group located farthest from the carboxyl group. On the other hand, this label has the nitroxyl group attached to carbon 16 (Figure 6), therefore, it could rotate in a region with less densely packed hydrocarbon chains because the major lipid in this organism has a hydrocarbon tail of only 16 carbon bonds and a few other lipids have longer chains. Similarly, the fact that, upon heat denaturation of the proteins, the motion of label [1,14] becomes more restricted and the environment more polar can be explained by either a stretching of the denatured proteins or by a compression of the width of the bilayer. At the moment, we have no clear way to distinguish between the two possibilities.

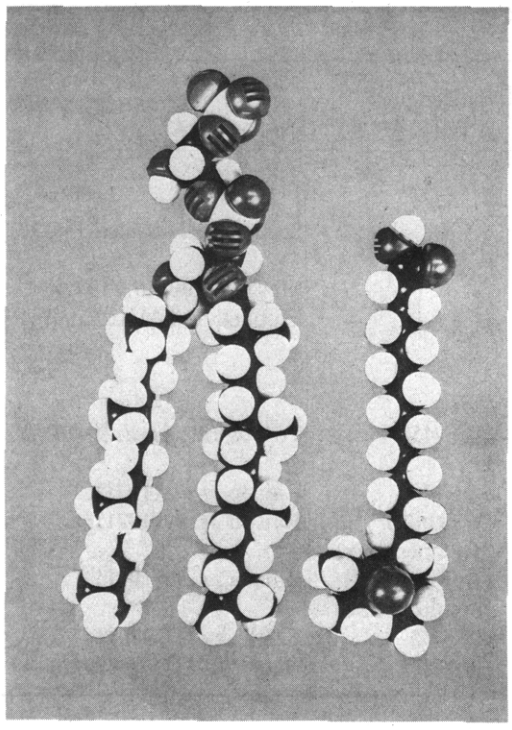

FIGURE 6: CPK models of spin-label [1,14] (right) and the major phospholipid of *H. cutirubrum*, 2,3-di-*O*-[3′,7′,11′,15′-tetramethylhexadecyl]glyceryl-1-phosphoryl-[1′′(3)-glyceryl 3′′(1′′)-phosphate] (left) (Kates *et al.*, 1965).

Numerous observations have been reported which demonstrate the effect of the physical state of the lipid phase on the activity of many membrane-bound enzymes (*e.g.*, Overath *et al.*, 1971; Raison *et al.*, 1971; Seelig and Hasselbach, 1971; Wilson *et al.*, 1970). However, reports are scarce on the reciprocal effect of proteins on lipids. While some authors (Henry and Keith, 1971; Rottem *et al.*, 1970; Tourtellotte *et al.*, 1970) propose very weak interactions between proteins and lipids, it seems that in *natural* membranes so far only for sarcoplasmic vesicles (Seelig and Hasselbach, 1971) and for *H. cutirubrum* membranes could strong protein–lipid interactions be shown. In the latter case, these interactions are not only evident from the results presented here, but also from earlier observations which indicate that the interaction of lipids with detergents is highly dependent on electron transport in these cells (Lanyi, 1972, 1973). Likewise, in *artificial* membranes it was shown very recently by Hong and Hubbell (1972) that a protein, rhodopsin, can inhibit segmental motions of the hydrocarbon chains in phospholipid bilayers. Earlier observations by Blaurock and Stoeckenius (1971), using the X-ray diffraction technique, and also spin-label studies (W. Stoeckenius and W. Hubbell, personal communication) have indicated that in a very specialized part of the membrane of another halophilic species, the purple membrane of *H. halobium*, the hydrocarbon chains can be extended and rigid. *H. cutirubrum*, the subject of this study, lacks this purple membrane.

According to a presently popular model for the structure of cell membranes (Singer and Nicholson, 1972), it is assumed that the cell matrix is a *fluid* lipid phase. However, as already pointed out by Oldfield and Chapman (1972), there is mounting evidence that in some membranes "rigid" as well as "fluid" domains can exist concomitantly and a balance between the two states may be of importance for function. If the spin labels detect only the most fluid regions (as discussed by these authors), then we have to say that *H. cutirubrum* membranes have only "rigid" lipid domains. It was also mentioned in this review that most organisms use steroids (*e.g.*, cholesterol) or hydrocarbons of different length and saturation to produce more rigid states. Since halophilic cells do not contain steroids (Kushawa *et al.*, 1972) and have only branched-chain hydrocarbons, it seems that in these organisms protein–lipid interactions may serve to achieve the same result.

Kreutz (1972) recently proposed a *general* model in which the coupling between the protein and lipid layers is accomplished by phosphatidylcholine (or chlorophyll in photosynthetic membranes). Yet in *H. cutirubrum* no phosphatidylcholine (nor chlorophyll) is present. While all these models cited fit certain biological membranes very well, it seems that the *H. cutirubrum* cell envelope membrane is unlike many others. A model for this membrane, consistent with our results, should include a protein matrix and hydrophobically bound, ordered lipids, in a manner similar to those proposed recently by Green and associates (Capaldi and Green, 1972; Vanderkooi and Green, 1971) and Steck *et al.* (1971).

Acknowledgments

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Control of Synthesis and Distribution of Acyl Moieties in Etiolated *Euglena gracilis*†

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ABSTRACT: In cell-free preparations of etiolated *Euglena gracilis* fatty acyl-coenzyme A (CoA) reductase was located mainly in the microsomes while incorporation of acetate into fatty acids occurred mostly in the 100,000*g* supernatant. The major labeled acyl chains in the wax ester fraction and the more polar lipids synthesized from [1-^{14}C]acetate by the soluble synthetase were C_{12},C_{14},C_{16}, and C_{16},C_{14},C_{12}, respectively, in the order of decreasing amounts. In the same order the small amount of labeled acyl chains formed by the mitochondria was C_{16}, C_{14}, and C_{20}. The major alcohol derived from [1-^{14}C]acetate by the soluble system was C_{16} while that formed by the mitochondria was C_{14}. Addition of 1-hexadecanol to the soluble synthetase caused a marked stimulation of incorporation of [1-^{14}C]acetate and of [2-^{14}C]-malonyl-CoA into the wax esters and an inhibition of the labeling of the polar lipids resulting in an overall increase in incorporation into total lipids. 1-Hexadecanol also changed the chain length distribution; at 0, 12.5, 25, and 50 μM added hexadecanol the major fatty acid synthesized was C_{12}, C_{10}, C_8, and C_6, respectively. Therefore, it is concluded that 1-hexadecanol, functioning as acyl acceptor, favored transfer of acyl chains formed by the synthetase to the alcohol and thus effectively competed with the subsequent condensation reaction. Cell-free preparations of *E. gracilis* readily converted C_{14} and C_{16} acids or their CoA esters into the corresponding fatty alcohols, whereas the fatty acids generated from [1-^{14}C]-acetate by the soluble synthetase were preferentially incorporated into the acyl portion of the lipids, suggesting a direct transfer of the acyl chains from the synthetase to the acyl acceptors. The microsomal preparations incorporated [2-^{14}C]malonyl-CoA into lipids. The major acyl chains formed by the microsomes were C_{16}, C_{14}, and C_{18} in order of decreasing amounts. In contrast to the soluble system, more than one-half of the label incorporated into lipids by the microsomes was in wax ester in which nearly 60% of the label was in the alcohol portion, suggesting that acyl chains which were reduced to alcohols may be synthesized by the microsomes themselves. The major labeled alcohol generated by the microsomes from malonyl-CoA was C_{16}. These results indicate that wax synthesis occurs in the microsomes by a complex of enzymes which synthesize acyl chains, reduce them, and esterify the alcohol to esters.

Regulation of fatty acid synthesis, mechanism and control of chain termination, and factors that control channeling of acyl chains into various classes of lipids including the reductive pathways involved in the synthesis of waxes and ether lipids are all poorly understood aspects of lipid metabolism. *Euglena gracilis* synthesizes many classes of lipids each containing its characteristic complement of acyl chains (Rosenberg, 1963, 1967; Guehler *et al.*, 1964). Furthermore, in this organism a large portion of acyl chains formed undergoes reduction to the alcohol. Therefore, *E. gracilis* is particularly suitable for determining the factors that control the synthesis and channeling of acyl chains into various pathways.

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