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CORRELATIONS BETWEEN FATTY ACID DISTRIBUTION IN PHOSPHOLIPIDS
AND THE TEMPERATURE DEPENDENCE OF MEMBRANE PHYSICAL STATE

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Running title: TEMPERATURE DEPENDENCE OF MEMBRANE PHYSICAL STATE

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ABSTRACT

Cytoplasmic membranes of an unsaturated fatty acid auxotroph of E. coli have been studied using spin-labeled hydrocarbon probes. These studies reveal that the membrane lipids undergo changes of state at critical temperatures which reflect the physical properties of the fatty acid supplement supplied to the cells during growth. The critical temperatures observed in spin labeled membranes correlate with characteristic temperatures in membrane functions. Lipid analysis reveals that fatty acid composition and distribution in membrane phospholipids^s are primary determinants of the temperatures at which changes of state are observed in membrane lipids. Fatty acid composition and distribution can also produce unique interactions between certain spin label probes and their lipid environment.

INTRODUCTION

The relationship between membrane lipid physical state and membrane function has been examined by a variety of techniques and approaches. Using bacterial auxotrophs (1-7), mycoplasma (8-10), and plant and animal mitochondria (11), several investigators have shown that many membrane functions are affected by lipid fatty acid composition. ~~These effects depend on and can be correlated with~~ composition. These effects depend on and can be correlated with the physical properties of the membrane lipids.

Fatty acid auxotrophs of E. coli provide an ideal system for investigating the relationship of membrane function to membrane lipid composition. The mutant used here and in a similar study (12) requires unsaturated fatty acids for growth, and also lacks the capacity to β -oxidize the supplements. It is thus possible to control closely the fatty acid components of the membrane lipids with the consequence that these mutants have a very simple lipid composition. In addition, two independent sugar transport systems of this mutant are well characterized with respect to their interrelated dependence on temperature and fatty acid supplement supplied for growth (4).

Recent electron spin resonance (ESR) studies on membranes and membrane lipids derived from this auxotroph indicate that the nonlinearities observed in Arrhenius plots for transport are a direct consequence of the physical state of the membrane lipids (12). Partitioning ~~of the nitroxide probe 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)~~ of the nitroxide probe 2,2,6,6-tetramethylpiperidine-1-oxyl (TEMPO)¹ between hydrophilic and hydrophobic phases of the membrane or isolated lipid suspensions revealed that there are two characteristic temperatures ~~marking changes in the physical state of the lipids (12).~~ marking changes in the physical state of the lipids (12). The higher of the two temperatures, t_h , marks the onset of "liquid" and "solid" phases in equilibrium. The lower temperature, t_l , marks the endpoint

of this equilibrium. The course of this equilibrium over the temperature range limited by t_h and t_z requires lateral phase separations of the membrane lipids (13). Above t_h , all lipids are in the liquid phase and below t_z , all lipids are in the solid phase. A careful examination of β -glucoside and β -galactoside transport has revealed two characteristic temperatures, t_h^* and t_z^* , for these functions, corresponding to t_h and t_z determined by TEMPO partitioning (12).

In the studies reported here, we have employed a different family of ESR probes, namely nitroxide labeled hydrocarbons. Use of these probes increases experimental sensitivity, with the result that it is possible to obtain interpretable spectra using relatively dilute aqueous membrane suspensions. In addition, we present a detailed analysis of the fatty acid distribution in the membrane lipids.

METHODS

Growth and Properties of Bacterial Strains.--Strain 30Ebox⁻

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 (14,15) and of this mutant are described elsewhere (12). Cells were cultured in a medium consisting of medium A (16) supplemented with 1% Difco casamino acids, 5 μ g per 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. Prior to harvesting, the cells were grown with the indicated essential fatty acid for at least 4 generations, and growth was followed turbidimetrically.

Induction and assay of transport, and isolation of inner (cytoplasmic) and outer membranes are described in detail elsewhere (4,15,17).

Extraction and Characterization of Lipids.--Lipids were extracted from membranes as previously described (18). Fatty acid methyl esters were obtained by transesterification with 8F₃-methanol (Applied ~~Science Laboratories~~ Science Laboratories), and the methyl esters were separated by gas-liquid chromatography on a 6 ft. x 1/8 in. column of 10% EGSSX on Chrom W DNCS.

Diglyceride analysis was performed by the method of Holub and Kuksis

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(19) with the omission of the acetylation step. Diglyceride species were eluted from silver nitrate impregnated thin layer plates by the method of Arvidson (20) and transesterified as described above.

RESULTS

Transport Studies.--Arrhenius plots of β -glucoside transport for cells grown with elaidic, oleic, and linoleic acid supplements all show two distinct changes in slope at characteristic temperatures (12). The lower characteristic temperature corresponds in each case to characteristic temperatures (t^* values) previously reported using the parent strain of that used here (2,7), as well as to temperatures observed using another spin label, TEMPO (12). Table 1 summarizes the physiological characteristic temperatures

for transport and the characteristic temperatures obtained using two different spin labels.

ESR Studies.--The mid-field to high field line height ratios obtained from spin labeled membrane preparations are shown as a function of temperature in Figs. 2, 3 and 4. These membranes are enriched in oleic, elaidic and linoleic acids respectively. The data show extrapolated intercepts occurring at approximately the same temperatures as those observed in β -glucoside transport (Table 1).

Similar results were obtained when more dilute membrane suspensions were used while the concentration of the spin label, 5N10, was held constant. These spectra gave splitting in the high field peak, resulting in two lines, indicating that 5N10 partitioned between the membrane hydrocarbon and polar zones. Plots of these data showing the ratio of the hydrocarbon high field line half-height to the polar high field line half-height gave intercepts similar to those shown in Figs. 2, 3 and 4. A higher characteristic temperature was observed only in linoleic acid enriched membranes. This was

detected with the spin labels, 5N10 and 6N11. The spin labels M12NS and vanadylacetylacetonate both detected the lower, but not the higher characteristic temperatures in the linoleate membranes.

Fatty Acid Analysis.--Lipids extracted from the β -oxidationless fatty acid auxotroph contain, as their only unsaturated fatty acid, the unsaturate supplied exogenously during growth (12). The overall fatty acid composition itself is simple and contains few components; consequently the phospholipids are composed of a relatively small number of species. Phosphatidyl-ethanolamine is the major phospholipid in E. coli (23-25).

Table 2 summarizes the distribution in diglyceride species of fatty acids derived from the phospholipids of inner membranes supplemented with oleate, linoleate and elaidate. The detailed distribution of individual fatty acids in diglycerides and in total lipid is shown in Table 3. Table 4 summarizes the percent of saturated and unsaturated fatty acids in diglycerides and in independent total membrane lipid samples. It is evident from these data that the fatty acid composition of the diglycerides faithfully reflects fatty acid composition of the total membrane phospholipids.

~~Lipids derived from cells supplemented with an elaidic acid supplement were quite distinctive. Over 80% of their fatty acid content was exogenously derived, and close to 70% of the diglycerides derived from phospholipid are of one molecular species. These data correlate well with those reported by Silbert (25), which showed that cells supplemented with a trans-mono-~~

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fitted with an X,Y recorder and operating at 9.5 GHz. A laboratory built variable temperature assembly was used which maintained a sample temperature tolerance of approximately $\pm 0.1^\circ\text{C}$. Temperatures were scanned in ascending order, and at least ten minutes was allowed for samples to come to thermal equilibrium at each temperature.

Two methods, illustrated in Fig. 1, were used to analyze the spectral data. The top spectrum, taken at 2°C , is of oleic acid enriched membranes labeled with 6N11. Two high field lines are visible and the basis of the data analysis is the ratio of the half-height of the peak representing spin label in a hydrophobic phase ($1/2 h_{1H}$) to the half-height of the peak representing spin label in a polar environment ($1/2 h_{1P}$). The bottom spectrum (Fig. 1), also taken at 2°C , is of a more concentrated sample of oleic acid enriched membranes. In this

~~bottom spectrum, the ratio of the heights of the mid-field (h_0) and high field (h_{-1}) lines is measured. This spectrum has the highest ratio in the temperature series as a consequence of the immobility of the spin label at this low temperature. Both methods of spectral analysis were used in evaluating the data discussed here and gave identical results.~~

Electron spin resonance is one of several spectroscopic techniques that have been used to examine thermal lipid phase transitions in biological membranes and model systems. It is a more sensitive technique than nuclear magnetic resonance, but less sensitive than spectrofluorimetry. The sensitivity of ESR of membranes, however, is dependent on the properties of the spin label probe. The spin label TEMPO, used in an independent study (12), is more water soluble than the hydrocarbon probes used here. This necessitated the use of concentrated membrane suspensions, usually in the form of pellets, to obtain workable signal-to-noise ratios. By virtue of its lipid solubility, TEMPO dissolves in the hydrocarbon phase of the membranes. When membrane lipids undergo a transition from a liquid to a more solid state, TEMPO is excluded from the hydrophobic environment and the spectrum reflects its presence in a more polar environment. The greatest resolution in the data obtained with TEMPO is in the high field, or third spectral line, where the hyperfine splitting indicates the partitioning of

the probe between hydrophobic and polar environments. The data obtained using TEMPO are thus analyzed on the basis of the line heights of these third line peaks.

The hydrocarbon probes used in the experiments described here have extremely limited aqueous solubility. They also bear a closer structural analogy than does TEMPO to fatty acids in membrane lipids. It was thus possible to use membrane suspensions on the order of a hundred-fold more dilute than in the studies where TEMPO was employed. The basis of the spectral analysis for experiments with the hydrocarbon probes is not only the detection of signal in hydrophobic versus polar environments, but also the restriction of motion of the spin label. The three spectral lines, illustrated in Fig. 1, would be of equal height and have a characteristic width and spacing for a spin label tumbling isotropically in a fluid environment. The line heights, widths and spacings change as the motion of the spin label is restricted by changes in environmental viscosity. These three parameters are qualitatively interrelated in reflecting changes in motion of the spin label. We have chosen to use the ratio of the heights of the mid-line and high field lines as a measure of the physical state of the environment of the spin label. This is the method of spectral analysis presented in the data here, although measurements of the polar and apolar third line heights gave identical results.

The spin label TEMPO, although the less sensitive of the two probes, yields more information. It permits detection of both the upper (tg_{hg}) and lower (tg_{lg}) characteristic temperatures which define the high and low temperature boundaries respectively of lateral phase separations. The hydrocarbon probes are more sensitive, requiring a lower concentration of membranes. They are thus more useful in applications where only small quantities of a preparation are available. Except in the case of linoleate membranes, however, the hydrocarbon probes do not detect tg_{hg} . On the basis of this information and other known properties of the interactions of

TEMPO and hydrocarbon probes with membranes, it is possible to speculate on the phenomena which give rise to the general inability of the hydrocarbon probes to detect t_{hg} .

When the temperature of an aqueous suspension of membranes labeled with TEMPO is lowered below t_{hg} , there is a progressive exclusion of TEMPO from the membranes into the aqueous environment. Conversely, when the temperature is raised above t_{lg} , TEMPO partitions into the hydrophobic regions of the membranes. This can be verified by sedimenting the membranes and assaying the TEMPO which appears in the supernatant (aqueous) fraction. With the hydrocarbon probes, on the other hand, the spin label remains associated with the membranes even below the lower characteristic temperature (the point at which virtually all membrane lipids will be in a "frozen" highly ordered state). In response to a temperature decrease, which gives rise to lateral phase separations (requiring lateral diffusion (13)), a spin label intercalated in a hydrocarbon environment can behave in one of two ways: 1) The spin label probe can be vertically expelled from the membrane. TEMPO may behave in this way since it has nearly equal solubility in the aqueous and liquid hydrophobic compartments of a membrane suspension. Thus a decrease in the volume of available hydrocarbon compartment concentration which arises as a consequence of ordering of hydrocarbon chains in lipids will result in a redistribution of TEMPO as expected from its partition coefficient. TEMPO would thus detect both the beginning and the end of lateral phase separations since the beginning and the end of lateral phase separations since its expulsion into the aqueous phase begins at t_{hg} and ends at t_{lg} .

2) The spin label can separate laterally within the hydrocarbon phase remaining with the solid or partitioning into the liquid phases. The hydrocarbon probes apparently behave in this manner. They are virtually insoluble in water and are thus not likely to be expelled from the membranes into an aqueous environment when the temperature is lowered. At the high membrane to spin label concentration ratio used here, the hydrocarbon probes may separate with the liquid hydrocarbon phase, since their structure

could give rise to deformation of a solid hydrocarbon phase. Therefore, little or no change in environment would be detected until the concentration of the liquid hydrocarbon phase is greatly reduced, i.e. at approximately tg_{1g} . Obviously, these hydrocarbon probes respond primarily to an event that occurs at tg_{1g} .

The above discussion concludes that in response to lateral phase separations, highly water soluble probes such as TEMPO will be expelled vertically from the membrane hydrocarbon phase. Probes which have low aqueous solubility, for example, the hydrocarbon probes, will engage in lateral phase separations in conjunction with the membrane lipids. The probes used in our study apparently preferentially partition laterally into the liquid hydrocarbon phase and usually detect only t_l , the point at which the concentration of the available liquid hydrocarbon phase approaches zero. Why then do the hydrocarbon probes also detect t_h in linoleate membranes? We have no definitive explanations for this, and can only identify the most obvious factor which could be causative. In the linoleate membranes there are two distinct major populations of phospholipids, disaturated and diunsaturated (see Table 2). The linoleate membranes are thus the only ones studied which have lipid classes with physical properties that are radically different from one another.

Several other groups of investigators have used fatty acid auxotrophs of E. coli to correlate the effects of lipid composition on the physical and physiological properties of membranes. Though there is general agreement that many membrane associated activities are affected by thermal phase transitions in membrane lipids, there are also inconsistencies between the data and the interpretations presented by these groups.

Some of these inconsistencies may have arisen since it was not known until recently that there are two characteristic temperatures (t_h and t_l) at which bulk lipid interactions can produce physiological "transitions" (i.e., intersects in multiphasic Arrhenius plots) in membrane function. In earlier studies, one of us reported physiological transitions in transport only at t_l (2,4,7). The failure to detect t_h was simply

we either did not study transport rate over a sufficiently broad temperature range to detect t_h , or did not assay for transport at a sufficient number of points in the temperature range studied in order to detect all the intersects. Overath and his colleagues have reported "transitions" in transport, monolayer physical chemistry of extracted lipids, and fluorescence properties of membranes and extracted lipids (3,24,26). They detected only a single transition in cells grown with each fatty acid supplement tested. In some cases (e.g., oleic acid supplemented cells), they reported a transition temperature that corresponds to our values for t_1 . In elaidate supplemented cells, however, their transition temperature corresponds to our value for t_h . There is as yet no explanation for this inconsistency.

Another source of possible confusion is that which could arise from the failure to distinguish "bulk" or "bilayer" lipid effects from "boundary" or "intimate" lipid effects. Esfahani, Wakil and their collaborators and Mavis, Bell and Vagelos have shown that a number of reactions catalyzed by membrane associated enzymes respond to lipid composition at temperatures other than t_h and t_1 (6,27). They have suggested that some proteins may have a selective affinity for certain lipid classes. This selectivity could be exerted on the basis of head group or fatty acid composition. Since head group and fatty acid composition can both influence thermal phase transitions (28-30), it is obvious that an "intimate" or "boundary" layer of lipids could produce a physiological transition distinct from t_h or t_1 if these "boundary" lipids had a composition different from that of the bulk of the membrane lipids. It is entertaining to make an analogy to the term "iceberg" which was coined by Klotz to characterize the interaction of proteins with an intimate shell of water (31). Perhaps it is appropriate to coin the term "lardberg" to refer to the interaction of a protein with its "intimate" or "boundary" lipid shell. This term is appropriate where a membrane protein exerts a rigidifying or stabilizing influence on an "intimate" lipid shell (32,33). Such a term should be used ^{with caution}.

however, since some lipid-protein interactions also might give rise to

local destabilization.

FOOTNOTES

¹ Other abbreviations used are: 5N10 (2,2-dimethyl,4-butyl,4-pentyl, N-oxyloxazolidine); 6N11 (2,2-dimethyl,4,4-dipentyl,N-oxyloxazolidine); and M12NS (2,2-dimethyl,4-hexane,4-methylundecanoate,N-oxyloxazolidine). (For structural formulas of 5N10 and 6N11, see Figures 2-4.)

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OFigure LEGENDS

FIGURE 1.

Top spectrum: The first derivative spectrum of a suspension of oleic acid enriched inner membranes ^(6 mg protein per ml) labeled with GNI1, taken at 2°C. Two high field lines are visible: hg_{-11lg} resulting from spin label localized in a nonpolar environment, and hg_{-1pg} resulting from label localized in a polar environment. See ^mmethods for description of data analysis.

Bottom spectrum: The first derivative spectrum of a suspension of oleic acid enriched inner membranes protein (16 mg protein per ml) labeled with GNI1, taken at 2°C. In this spectrum, the ratio of the line heights of the mid-field (hg_{0g}) to high field (hg_{-1g}) is measured.

See meth

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~~See Methods for further details.~~

See Methods for further details.

FIGURE 2

A suspension of inner membranes (10 mg protein per ml) derived from oleate grown cells was labeled with 6N11 (structure shown on figure). ESR spectra were taken over the temperature range indicated. The ratio of the mid-line and high field line heights is plotted as a function of the reciprocal of the absolute temperature.

FIGURE 3

A suspension of inner membranes (2.5 mg protein per ml) derived ~~from oleate~~ from elaidate grown cells was labeled with 6N10 (structure shown on figure). ESR spectra were taken over the temperature range indicated. The ratio of the mid-line and high field line heights is plotted as a function of the reciprocal of the absolute temperature.

FIGURE 4

A suspension of inner membranes (5 mg protein per ml) derived from linoleate grown cells was labeled with 6N11. ESR spectra were taken over the temperature range indicated. The ratio of the mid-line and high field line heights is plotted as a function of the reciprocal of the absolute temperature.

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TABLE 1. CHARACTERISTIC TEMPERATURES FOR GLUCOSIDE TRANSPORT AND SPIN LABELS.

Fatty acid supplement	System:	t_h^* Transport	t_h TEMPO	t_h Hydrocarbon	t_l^* Transport	t_l TEMPO	t_l Hydrocarbon
Elaidate		38.6-38.8	37.7	--	32.1	30.7	30.3
Oleate		26.0 & 21.8	31.0	--	14.4	15.8	16.2
Linoleate		27.1	28.5	28.4 $\Rightarrow$	6.8	8.9	9.1

The characteristic temperatures for b-glucoside transport and TEMPO ⁰ Partitioning are described elsewhere (12). The characteristic temperatures observed with the spin labeled hydrocarbons 5N10 and 6N11 were obtained as described in Methods. The high temperature boundary of the lateral phase separations is denoted t_h , and the low temperature boundary, t_l . Discontinuation ^{ities} in Arrhenius plots of b-glucoside transport vs $1/T$ which correlate with t_h and t_l are denoted t_h^* and t_l^* .

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TABLE 2. DISTRIBUTION OF SATURATED AND UNSATURATED FATTY ACIDS
IN DIGLYCERIDES DERIVED BY ENZYMIC DIGESTION FROM INNER
MEMBRANE PHOSPHOLIPIDS

Fatty acid supplement for growth	Disaturated	Saturated:Unsaturated	Diunsaturated
Oleate	1.2%	77.1%	19.7%
Linoleate	40.8%	19.8%	39.4%
Elaidate	50.1%	31.3%	68.7%

An extract of inner membrane phospholipids (Methods) was digested with Clostridium welchii phospholipase C. Comparison of the starting and digested materials revealed complete digestion of phosphatidyl-ethanolamine and phosphatidylglycerol (which account for more than 98% of the total phospholipids in inner membrane derived from the strain of E. coli used here [Machtiger and Fox, unpublished results]). The diglycerides were resolved as described in Methods. The fatty acid content in each diglyceride was determined by gas-liquid chromatography (Methods). The total fatty acids derived from the sum of all fatty acids in the resolved, transesterified diglycerides was assigned a value of 100%, and the percent distribution of diglycerides was calculated on this basis.

308

~~the percent distribution~~

TABLE 3. COMPARISON OF FATTY ACID COMPOSITION OF TOTAL INNER MEMBRANE PHOSPHOLIPIDS WITH THAT OF RESOLVED DIGLYCERIDES (TABLE 2) DERIVED FROM THESE LIPIDS

Fatty acid supplement for growth	Sample	Fatty acid						
		14:0	16:0	16:1	18:0	18:1	18:2	Other
Oleate	Diglycerides	2.9	36.1	0.8	0	60.2	--	--
	Membrane lipid	6.5	38.5	2.0	--	51.0	--	2.0
Linoleate	Diglycerides	4.3	45.5	1.9	<1.0	5.6	40.6	1.1
	Membrane lipid	2.9	44.0	1.9	--	--	48.9	2.3
Elaidate	Diglycerides	2.1	10.7	<0.1	<1.0	86.4	--	--
	Membrane lipid	4.5	8.7	--	--	86.4	--	--

Fatty acid chain length and degree of unsaturation are denoted by numbers before and after the colon respectively. No difference in retention time in gas-liquid chromatography is detected for cis- Δ^9 -C18:1 (oleic acid) and trans- Δ^9 -C18:1 (elaidic acid) by the methods used here.

TABLE 4. COMPARISON OF SATURATED AND UNSATURATED FATTY ACIDS IN TOTAL INNER MEMBRANE LIPIDS AND IN RESOLVED DIGLYCERIDES DERIVED FROM THESE LIPIDS

Fatty acid supplement for growth	Saturated fatty acids		Unsaturated fatty acids		Ratio: saturated/unsaturated	
	Total lipid	Diglycerides	Total lipid	Diglycerides	Total lipid	Diglycerides
Oleate	45.0*	39.7	53.0*	58.2	0.85	0.68
Linoleate	46.9 [†]	50.7	50.8 [†]	49.3	0.92	1.03
Elaidate	13.2	15.6	86.8	84.3	0.15	0.18

Other: 2.0%*; 2.3%[†].

Analyses of inner membrane total lipids and diglycerides were performed on independently derived samples as described in Methods. The distribution of saturated and unsaturated fatty acids in total lipid was obtained by simple summation. The ratio of saturated and unsaturated fatty acids in diglycerides was calculated from the data in Table 3.

FIG-1

FIG 2

FIG 3

PLA/B

FIG. 4

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