Physical Properties of Membranes Isolated from Tissue Culture Cells with Altered Phospholipid Composition*

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A choline-requiring strain of mouse fibroblast cells (LM cells) was cultured in suspension with choline, N,N'-dimethylethanolamine, N-monomethylethanolamine, or ethanolamine. These choline analogues were incorporated into membrane phospholipids as phosphatidyl-N,N'-dimethylethanolamine, phosphatidyl-N-monomethylethanolamine, and phosphatidylethanolamine. Plasma membranes, microsomes, mitochondria, and their respective lipids were isolated and the characteristic temperatures were determined by using two types of fluorescent probes: (a) β-parinaric acid, a naturally occurring molecule, and (b) 8-anilino-1-naphthalene sulfonic acid, a synthetic organic fluorophore. A computer-centered spectrofluorimeter capable of simultaneous measurement of absorbance, absorbance-corrected fluorescence, and relative fluorescence efficiency was utilized for on-line measurement of all fluorescence parameters. Plots of absorbance corrected fluorescence or of relative fluorescence efficiency versus temperature revealed the same five characteristic temperatures with both types of probe. These characteristic temperatures were independent of the phospholipid composition of the LM suspension cell membranes or their extracted lipids. Plasma membranes, microsomes, and mitochondria containing analogue phospholipids had similar (±1°C) characteristic temperatures.

The presence of analogue phospholipids altered the binding characteristics of β-parinaric acid with plasma membranes and plasma membrane lipids of LM suspension cells. The equilibrium dissociation constant of plasma membranes and plasma membrane lipids was decreased 2- and 5-fold, respectively, when the cells had been supplemented with ethanolamine. The minimum number of phospholipid molecules per probe binding site was approximately constant in the intact plasma membrane but increased (2-fold) in the isolated plasma membrane lipids. The presence of analogue phospholipids also altered the interaction of 8-anilino-1-naphthalene sulfonic acid with LM cell membranes. The equilibrium dissociation constant of this probe interacting with mitochondria was decreased about 10% when LM cells were supplemented with ethanolamine. Similarly, the equilibrium dissociation constant of 8-anilino-1-naphthalene sulfonate interacting with mitochondrial lipid was decreased 40% by ethanolamine supplementation. The fluorescent properties of both probes were sensitive to the degree of methylation of the polar head group. The absolute values of absorbance-corrected fluorescence and relative fluorescence efficiency were different for each type of membrane from LM cells even with the same analogue supplement. Thus, it appears that LM cells maintain the characteristic temperatures which are a measure of the physical properties of their membranes, despite large alterations of the phospholipid polar head group composition.

Membranes of cells are in a fluid state and recently direct evidence for membrane fluidity has been presented (1). The physical properties of pure lipids have been studied extensively in order to explain the transitions in physical properties observed with probe molecules in naturally occurring membranes or their isolated heterogeneous lipids (2-12). It was expected that individual transitions might be ascribed to particular lipid classes. Instead a vast polymorphism has been indicated for phospholipids of mitochondria (13), brain (14, 15), egg yolk (15, 16), and neutral lipids (17). The exact transition temperature from the gel to liquid crystalline phase of a phospholipid appears to be dependent on as many as nine different variables (15). Even binary mixtures of pure phospholipid species give two or sometimes three characteristic temperatures. Such data have been interpreted as indicating the
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The results of our investigations detail the isolation of subcellular fractions and the lipid composition thereof and compare the activities of membrane-bound enzymes as a function of polar head group composition of the phospholipids. It was shown that the activities of seven membrane-bound enzymes from LM cell membranes were independent of polar head group composition and that several compensatory mechanisms may exist for maintaining the physical characteristics of the membranes. We have previously shown that five characteristic temperatures exist in plasma membrane, microsomes, mitochondria, and their lipids from LM cells grown in suspension culture. Herein are presented the results of investigations indicating that the characteristic temperatures of the LM cell membranes and isolated membrane lipids are independent of the phospholipid composition. Several interpretations of these data are presented.

MATERIALS AND METHODS

Cell Culture—LM cells, a choline-requiring strain of mouse fibroblasts, were grown in suspension culture on the chemically defined, lipid-free, serum-free medium of Higuchi (43) modified as described.

Choline analogue supplementation was carried out by washing log phase LM cells two times with choline-deficient medium. The cells were pelleted in an International PR-1 centrifuge for 5 min at 1000 rpm after each resuspension. Aliquots of the washed cells were then resuspended at 1 x 10^6 cells/ml in fresh medium and one of the following was added at 40 μg/ml: choline, N,N-dimethyl ethanolamine, N-monomethyl ethanolamine, or ethanolamine. After 2 days growth at 37°C the cells were diluted with fresh medium containing the appropriate analogue in order to maintain the cells growing in log phase. Then after 1 additional day of growth at 37°C (3 days total) the cells were harvested and plasma membranes, microsomes, mitochondria, and their respective extracted lipids were obtained as described previously. Choline, N,N-dimethyl ethanolamine, N-monomethyl ethanolamine, and ethanolamine were obtained from Eastman Kodak Co., Rochester, N.Y.

Fluorescence Spectroscopy and Sample Preparations—The computer-centered spectrofluorimeter described by Holland and co-workers (44, 45) was used for all measurement. β-Parinaric acid was incorporated into LM suspension cell membranes or lipids as described previously. ANS (8-anilino-1-naphthalene sulfonic acid) was obtained from Pierce Chemical Co., Rockford, III. as the ammonium salt. Stock solutions of ANS were prepared fresh daily at 1 to 5 x 10^-3 M in distilled H_2O. Aliquots of this stock solution were added directly to membranes (50 μg of protein/ml of phosphate-buffered saline, pH 7.4) or to lipid extracts. The samples were then blended for 5 min on a Vortex Genie mixer at maximum speed, followed by a 30-min incubation at 45°C (46). The samples were then blended on a Vortex mixer for 1 min and placed in the fluorimeter sample cuvette and spectral measurements were determined as described.

Plots of CO_{2} of ANS versus temperature in solvents such as ethanol indicated an exponential decay with increasing temperature. No discontinuities or characteristic temperatures were formed. Thus the characteristic temperatures determined under “Results” with membranes or lipids do not appear to be systematic instrumental artifacts.

RESULTS

Effect of Choline Analogue Supplementation on Characteristic Temperatures Monitored with β-Parinaric Acid. The characteristic temperatures of LM membranes or lipids containing large amounts of phosphatidyl-N,N'-dimethyl ethanolamine, phosphatidyl-N-monomethyl ethanolamine, or phosphatidyl-ethanolamine may be different from those containing primarily phosphatidylcholine if no other changes in lipid composition

The abbreviations used are: LM cells, a choline-requiring strain of mouse fibroblast cells; ANS, 8-anilino-1-naphthalene sulfonic acid; CO, absorption-corrected fluorescence; RFE, relative fluorescence efficiency.

1 The abbreviations used are: LM cells, a choline-requiring strain of mouse fibroblast cells; ANS, 8-anilino-1-naphthalene sulfonic acid; CO, absorption-corrected fluorescence; RFE, relative fluorescence efficiency. Schroeder, F., Holland, J. F., and Vagelos, P. R. (1976) J. Biol. Chem. 251, 5015-5026.

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occurred. In order to test this possibility it was necessary that a probe molecule sensitive to the polar head group composition be utilized. The naturally occurring fluorescence molecule, β-parinaric acid, was chosen as a probe for this study because: (a) it satisfied many of the criteria for an optimal probe molecule previously set forth (10, 11), (b) β-parinaric acid has been used to determine the transition temperatures of model lipids from the gel to liquid crystalline state. Sklar et al. (Ref. 11 and Footnote 5) demonstrated that the fluorescence of β-parinaric acid was sensitive to both acyl chain length and polar head group composition of phospholipids. The transition temperatures of synthetic phospholipids reported by the β-parinaric acid fluorescence were almost identical to those obtained with differential scanning calorimetry6 (7, 8). Tables I, II, and III show the characteristic temperatures reported by β-parinaric acid for plasma membranes, microsomes, mitochondria, and their respective extracted lipids isolated from LM suspension cells supplemented with choline, N,N'-dimethylethanolamine, N-monomethylethanolamine, or ethanolamine. The characteristic temperatures were almost identical (±1°) to those previously obtained with choline-supplemented cells. The location of excitation and emission maxima were not altered by the presence of different analogue phospholipids in the membranes. These experiments were repeated at four different concentrations (20-fold range) and similar characteristic temperatures (±1°) were obtained in both ascending and descending temperature scans.

Relative fluorescence efficiency (RFE) is an inherent property of a fluorophore in a particular environment and is independent of solubility changes. Both parameters indicated the same characteristic temperatures.

### Table I

**Characteristic temperatures of plasma membranes and extracted lipids from LM cells supplemented with choline analogues**

| Supplement          | Spectral parameter | Characteristic temperatures | Plasma membranes | Plasma membrane lipids |
|---------------------|--------------------|-----------------------------|------------------|------------------------|
| Choline             |                    | 19° 23° 31° 37° 43° 49° 55° | 17° 23° 30° 37° 44° |
| N,N'-Dimethylethanolamine |                    | 19° 23° 31° 37° 43° 49° 55° | 17° 23° 30° 37° 44° |
| N-Monomethylethanolamine |                    | 19° 23° 31° 37° 43° 49° 55° | 17° 23° 30° 37° 44° |
| Ethanolamine        |                    | 19° 23° 31° 37° 43° 49° 55° | 17° 23° 30° 37° 44° |

### Table II

**Characteristic temperatures of microsomes and extracted lipids from LM cells supplemented with choline analogues**

| Supplement          | Spectral parameter | Characteristic temperatures | Microsomes | Microsome lipids |
|---------------------|--------------------|-----------------------------|------------|-----------------|
| Choline             |                    | 19° 23° 31° 37° 43° 49° 55° | 17° 23° 30° 37° 44° |
| N,N'-Dimethylethanolamine |                    | 19° 23° 31° 37° 43° 49° 55° | 17° 23° 30° 37° 44° |
| N-Monomethylethanolamine |                    | 19° 23° 31° 37° 43° 49° 55° | 17° 23° 30° 37° 44° |
| Ethanolamine        |                    | 19° 23° 31° 37° 43° 49° 55° | 17° 23° 30° 37° 44° |

### Table III

**Characteristic temperatures of mitochondrial membrane and extracted lipids from LM cells supplemented with choline analogues**

| Supplement          | Spectral parameter | Characteristic temperatures | Mitochondria | Mitochondria lipids |
|---------------------|--------------------|-----------------------------|--------------|--------------------|
| Choline             |                    | 19° 23° 31° 37° 43° 49° 55° | 17° 22° 30° 37° 44° |
| N,N'-Dimethylethanolamine |                    | 19° 23° 31° 37° 43° 49° 55° | 17° 22° 30° 37° 44° |
| N-Monomethylethanolamine |                    | 19° 23° 31° 37° 43° 49° 55° | 17° 22° 30° 37° 44° |
| Ethanolamine        |                    | 19° 23° 31° 37° 43° 49° 55° | 17° 22° 30° 37° 44° |
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It is possible that at least some of these alterations in fluorescence parameters of β-parinaric acid were due to changes in binding ability of the probe to various phospholipids (46, 47). It has been shown that the dissociation constant, $K_D$, and the minimum number of phospholipid molecules providing one binding site for β-parinaric acid in LM suspension cultured cell plasma membranes or plasma membrane lipids varied only slightly with temperature. As indicated in Table IV, the $K_D$ and the size of the phospholipid binding site for β-parinaric acid were affected by the polar head group composition of the plasma membrane phospholipid. Plasma membrane lipids from LM suspension cells supplemented with $N,N'$-dimethylethanolamine and plasma membranes and lipids from cells supplemented with $N$-monomethylethanolamine or ethanolamine had smaller $K_D$ values and therefore higher affinities for β-parinaric acid. The plasma membrane lipids of choline supplemented cells had a slightly higher $K_D$ for β-parinaric acid than did the intact membrane (1.04 versus 0.93 μM). Supplementation with analogues such as $N,N'$-dimethylethanolamine, $N$-monomethylethanolamine, or ethanolamine reversed this trend since the plasma membrane lipids had 2- to 3-fold smaller $K_D$ values for β-parinaric acid than the intact plasma membranes. The minimum number of phospholipid molecules per binding site increased slightly in the plasma membranes (with the exception of ethanolamine supplementation), while it increased 2- to 3-fold in the plasma membrane lipids, depending on the analogue supplemented. Thus, increased affinity for the probe was accompanied by increased numbers of phospholipid molecules per binding site.

Further, it appears that the absolute values of $CO_{115}$ may depend on the type of analogue present in the membrane. The presence of analogue phospholipids may result in differences in the amount of probe that can be bound to the membrane or its isolated lipids. These differences in the level of probe saturation may be responsible for the observed differences for β-parinaric acid in membranes of different phospholipid composition. However, as stated earlier, nonsaturating concentrations of β-parinaric acid were used for all membranes and isolated membrane lipids studied here. Such criticisms would not be valid for relative fluorescence efficiency (RFE) which does not depend on concentration of the probe. This parameter, RFE$_{115}$, also indicated the same characteristic temperatures as $CO_{115}$.

**Determination of Characteristic Temperatures with Other Fluorescence Probes**—An objection to the results obtained with β-parinaric acid might be that we have measured some inherent properties of the probe, β-parinaric acid, that do not reflect transitions or membrane properties in our system. Therefore, the above experiments were repeated with the fluorescence probe ANS. ANS is an organic molecule not occurring in nature; it is water-soluble and fluoresces weakly in aqueous environments (31, 46). The fluorescence characteristics of ANS in a variety of solvents and in LM cells membranes or lipids are shown in Table V. The spectral characteristics (emission maximum and excitation maxima) of ANS in the plasma membranes, mitochondria, or their respective lipids were similar to those when the probe was present in increasingly hydrophobic solvents. ANS will bind to both proteins as well as lipids (10, 23, 31, 46) and the membrane data presented here represent average values possibly reflecting contributions from both of these membrane components. The increases in the relative fluorescence efficiency (RFE$_{115}$) of ANS with different solvents (methanol and ethanol) were similar to published increases of absolute quantum efficiency using other instruments and different salts of the probe (23, 31, 48). The fluorescence efficiency of ANS in plasma membrane lipids decreased significantly when compared to the intact membranes. This behavior is opposite to that of β-parinaric acid which showed a slight (10%) increase in RFE$_{115}$ in the isolated lipid. ANS relative fluorescence efficiency increased 118-fold and 24-fold in the presence of plasma membranes and mitochondria, respectively, the ratio of RFE in the plasma membrane to the RFE in the mitochondria being approximately 4. The increase in relative fluorescence efficiency with the respective isolated lipids was much smaller (11- and 16-fold) but the ratio between the two was also close to 4. Similar ratios of RFE in the plasma membrane to the RFE in the mitochondria were also noted with β-parinaric acid in both the membrane and in the isolated lipids. As with β-parinaric acid, the emission and excitation maxima of ANS did not change with membranes or lipids containing different phospholipids (plasma membranes versus mitochondria). These spectral alterations are characteristic for the behavior of ANS when present in solvents of low polarity as well as on binding to lipids, proteins, or cellular membranes (10). Since 8-anilino-1-naphthalene sulfonate can interact with both proteins and lipids, the portion of plasma membrane lipids apparently accessible to ANS can be determined as shown in Table VI by a previously published method (28). As indicated near two possible transitions, the fraction of plasma membrane lipids

**Table IV**

**Binding characteristics of β-parinaric acid with plasma membrane and plasma membrane lipids of LM cells supplemented with choline analogues**

| Fraction      | Supplement             | $T$  | $K_D$ ($\mu$M) | Minimum number of lipid molecules/ binding site$^a$ |
|---------------|------------------------|------|----------------|-----------------------------------------------|
| Plasma membranes | Choline                | 24*  | 0.93           | 11                                           |
|                | $N,N'$-Dimethylethanolamine | 24  | 0.94           | 22                                           |
|                | $N$-Monomethylethanolamine | 24  | 0.78           | 19                                           |
|                | Ethanolamine           | 24  | 0.50           | 16                                           |
| Plasma membrane lipids | Choline                | 24  | 1.04           | 15                                           |
|                | $N,N'$-Dimethylethanolamine | 24  | 0.35           | 30                                           |
|                | $N$-Monomethylethanolamine | 24  | 0.39           | 38                                           |
|                | Ethanolamine           | 24  | 0.22           | 40                                           |

$^a$ $K_D$, the equilibrium dissociation constant was determined as previously described (23).

$^b$ The minimum number of phospholipid molecules providing one binding site was estimated as previously described (23).
Plasma membrane lipids

The presence of choline. As previously indicated with P-parinaric acid, the alterations in the absorbance-corrected fluorescence (COssr) could be due to changes in binding affinity differences. However, the relative fluorescence efficiency, RFEssr, a concentration-independent parameter, indicated the same characteristic temperatures as COssr for ANS in LM cell membranes and lipids.

The effect of base analogue supplementation and thereby altered phospholipid composition of LM cell membranes on characteristic temperatures was investigated with ANS. As shown in Tables VII and VIII, the characteristic temperatures were almost identical (+1°) for plasma membranes, mitochondria, or their isolated lipids. Both COssr and RFEssr indicated that altered phospholipid composition had little if any effect on the characteristic temperatures of either membranes or lipids. It is also possible that differences in phospholipid compositions could result in changes in ANS binding affinity to the LM cell membranes and cause decreases in COssr as shown in Figs. 1 and 2. Again equilibrium dissociation constants were determined to test this possibility. In the case of mitochondria the Kn values at 24° were 30, 30, 27, and 27 for choline-, N,N'-dimethylethanolamine-, N-mononethylethanolamine-, and ethanolamine-supplemented cells, respectively. The isolated mitochondrial lipids had lower Kn values at 24° (11, 8, 9, and 7 μM, respectively). Therefore the analogue phospholipids increased binding affinity for ANS. These results indicated that drastically lowered values of COssr with different base analogues were not primarily due to greatly decreased binding affinities of ANS for the membranes or lipids.

The results reported by ANS with membranes from LM cells supplemented with different choline analogues (Tables VII and VIII) illustrated that the characteristic temperatures indicated by ANS were not altered by the presence of analogue phospholipids in either the membranes or their extracted lipids. Thus the results obtained with ANS agree with those obtained by β-parinaric acid despite considerable differences in the nature of the probes, their affinity for lipid, and their location in the membranes.

Effect of Lipid Environment on Fluorescence Properties of Probes in LM Cell Membranes and Membrane Lipids—The affinity of the LM cell lipids and membranes for the probes β-parinaric acid and ANS has been documented. Hence an investigation concerning the absolute values of CO and RFE as a function of polar head group methylation appeared desirable. The results of these studies are shown with β-parinaric acid in plasma membranes, microsomes, mitochondria, and their

The limiting corrected fluorescence at 364 nm (ΔCOssr) was determined near two possible transitions for plasma membranes and plasma membrane lipids as previously described (23). The accessible fraction is defined (23) as the value of ΔCOssr in the plasma membrane divided by the corresponding value in the plasma membrane lipids. Accessible to ANS appears to be approximately 46%. A similar figure for β-parinaric acid was approximately 92%. Thus, if it is assumed that β-parinaric acid can interact with the maximal amount of lipid present, then ANS is capable of interaction with about 50% of available plasma membrane lipid. The remainder of the probe fluorescence may then be due to interaction with other molecules such as proteins or possibly to some particular interaction that requires a particular microenvironment present only in the intact membranes. Thus, there are some fundamental differences as well as some similarities in spectral behavior of ANS versus β-parinaric acid when each was used as a membrane probe.

The characteristic temperatures of plasma membranes, mitochondria, and their isolated lipids were determined with 8-anilino-1-naphthalene sulfonate. Absorbance-corrected fluorescence (COse) and relative fluorescence efficiency (RFEse) were plotted versus temperature as shown in Figs. 1 and 2. Five characteristic temperatures very similar to those noted with β-parinaric acid were indicated by both spectral parameters in membranes as well as lipids obtained from LM cells grown in the presence of choline. As previously indicated with β-parinaric acid, the alterations in the absorbance-corrected fluorescence (COse) could be due to changes in binding ability of the probe. Such alterations have been shown to be a function of temperature with ANS (47). Therefore, the equilibrium dissociation constants, Kn, were determined as previously described (23). The Kn of ANS in LM suspension cell plasma membranes at 20° was 23 μM, while at 40° it was 12 μM as compared to the relatively constant Kn of β-parinaric acid over this temperature range. Similar alterations were observed with

### Table V

| Environment          | ANS Emission maximum | Exsitation maximum | RFE<sup>a</sup>  | Increase in RFE relative to PBS | Absolute quantum efficiency<sup>a</sup> | Increase in absolute quantum efficiency relative to PBS |
|----------------------|-----------------------|--------------------|------------------|---------------------------------|----------------------------------------|------------------------------------------------------|
| PBS                  | 3.33                  | 555                | 262, 344         | 0.99                            | 0.004                                  | 55                                                   |
| Methanol             | 3.33                  | 480                | 264, 364         | 69.1                            | 70                                     | 0.22                                                 |
| Ethanol              | 3.33                  | 480                | 265, 365         | 112                             | 113                                    | 0.37                                                 |
| Plasma membrane      | 3.12                  | 480                | 265, 365         | 117                             | 117                                    | 0.37                                                 |
| Plasma membrane lipids | 4.18              | 480                | 265, 365         | 117                             | 118                                    | 0.37                                                 |
| Mitochondria         | 4.16                  | 480                | 265, 365         | 70                              | 71                                     | 0.37                                                 |
| Mitochondria lipids  | 4.16                  | 480                | 265, 365         | 16                              | 16                                     | 0.37                                                 |

<sup>a</sup> The RFE was determined at the excitation maximum with longest wavelength.

<sup>b</sup> PBS, phosphate-buffered saline.

<sup>c</sup> These values were taken from previously published data of Stryer et al. (50).

### Table VI

| Cell fraction                | ΔCO<sub>ssr</sub>* near possible transition: | 24–30° | 37.43° |
|-----------------------------|---------------------------------------------|--------|--------|
| Plasma membrane             | 3.57                                        | 1.70   |        |
| Plasma membrane lipids      | 7.70                                        | 3.70   |        |
| Accessible fraction         | 0.46                                        | 0.47   |        |

<sup>*</sup> The limiting corrected fluorescence at 364 nm (ΔCO<sub>ssr</sub>) was determined near two possible transitions for plasma membranes and plasma membrane lipids as previously described (23). The accessible fraction is defined (23) as the value of ΔCO<sub>ssr</sub> in the plasma membrane divided by the corresponding value in the plasma membrane lipids.
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FIG. 1. Effect of temperature on spectral parameters of ANS in plasma membranes and extracted plasma membrane lipids. As described under "Materials and Methods," ANS (final concentration, 4.16 μM) was interacted with LM cell plasma membranes and lipids, CO₃₄ and RFE₃₄ were determined, emission was monitored at 480 nm, and temperature was varied at 2°C/min and monitored.

FIG. 2. Effect of temperature on spectral parameters on ANS in mitochondria and extracted mitochondrial lipids. All methods as described in legend of Fig. 1.

isolated lipids at three different temperatures (see Figs. 3 to 5). Several major trends were evident. (a) Decreases or increases in CO₃₄ and RFE₃₄ of β-parinaric acid as a function of degree of polar head group methylation were in the same direction in both membranes and lipids. (b) The CO₁₃ and RFE₁₃ indicated similar trends at three different temperatures. (c) Lastly, the probe behaved differently in each particular membrane or membrane lipid as a function of the number of CH₃ groups on the polar head group nitrogen of the phospholipid analogue. The absolute values of CO₁₃ and RFE₁₃ decreased in plasma membranes and increased in microsomes with decreasing polar head group methylation. The value for mitochondria did not illustrate any consistent trend. The lipid composition of each of the above membranes isolated from LM suspension cells was quite different, and the distribution of analogue phospholipids also varied between the three membranes. Similar results were obtained when these experiments were repeated with ANS as shown in Figs. 6 and 7.

DISCUSSION

Previous results (11, 15) indicate that the characteristic temperatures obtained with fluorescent probes are sensitive to the acyl as well as polar head group composition of phospholipids. The polar head groups of membrane lipids of LM cells grown in suspension culture with choline analogues were altered such that up to 50% of membrane phospholipids were analogue phospholipid. The data presented here indicate that the characteristic temperatures of such membranes or their lipids were unaltered. Characteristic temperatures, or transitions, are believed to reflect changes in the physical states of lipids in membranes (1-4, 6-12, 23-29). If it is assumed that only changes in polar head groups occur on supplementing LM cells with base analogues, our results would be contrary to expectations of increased transition temperatures with decreasing numbers of methyl groups on the nitrogen of phospholipids as has been shown in model membranes (7, 8, 11). It was previously shown by differential scanning calorimetry as well as by fluorescence probe analysis with β-parinaric acid that the transition temperatures of a series of phospholipids with identical acyl substituents increased linearly with decreasing numbers of methyl groups on the nitrogen atom of the phospholipid base (7, 8, 11). The difference in transition temperatures between diaclylphosphatidylcholine and an identical diacylphosphatidylethanolamine can be as much as 30°C with acyl groups 18 carbons long (8, 11) or 60°C with acyl groups 12 or 14 carbons long (15). Therefore, the results obtained with LM cell membranes and whole LM cells (40-42) in which up to 50% of the phospholipids were analogue phospholipids that were incorporated in vivo were indeed surprising. We postulate that the LM cells must be compensating for changes in polar head groups of lipids in order to maintain a constancy of the lipid physical properties as indicated by the characteristic temperatures.

A variety of mechanisms for maintaining the physicochemical properties of membranes may be available to eukaryotes. Several of these mechanisms have been discussed in detail previously. One additional compensating mechanism may be important for LM cell response to choline analogue supplementation. In LM suspension cell membranes the percentage of both phosphatidylcholine and phosphatidylethanolamine decrease in the presence of choline analogues. These altered ratios themselves may help to compensate for the introduction of new phospholipids with different phase transition temperatures and theoretical considerations of this point may be presented. Table IX indicates the theoretical transition temperatures, Tₜ, of phospholipid from LM suspension cells calculated from a series of dipalmitylphosphatides. The actual values of each diaclylphosphatide were taken from data of others (7, 8, 11) and were multiplied by the mole fraction of that phosphatide in the LM phospholipids. The sum of such partial contribution to the transition temperature was taken for the four major phosphatides indicated in the table and presented in the last column. The major assumptions in these
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**TABLE VII**

Characteristic temperatures of plasma membranes and extracted lipids from LM cells supplemented with choline analogues

Characteristic temperatures were obtained with ANS (4.16 μM) as stated under "Materials and Methods."

| Supplement               | Spectral parameter | Characteristic temperatures | Plasma membranes | Plasma membrane lipids |
|--------------------------|--------------------|-----------------------------|-------------------|------------------------|
|                          |                    |                             |                   |                        |
| Choline                  | CO<sub>254</sub>   | 20° 24° 32° 36° 39° 20° 24° 30° 37° 42° |                   |                        |
|                          | RFE<sub>254</sub>  | 20 23 31 36 40 20 25 29 36 44   |                   |                        |
| N,N'-Dimethylethanolamine| CO<sub>254</sub>   | 19 25 30 38 41 19 24 29 36 40   |                   |                        |
|                          | RFE<sub>254</sub>  | 19 24 31 38 43 19 23 30 37 42   |                   |                        |
| N-Monomethylethanolamine | CO<sub>254</sub>   | 19 25 32 37 42 19 24 30 38 43   |                   |                        |
|                          | RFE<sub>254</sub>  | 21 26 32 37 42 20 26 32 37 42   |                   |                        |
| Ethanolamine             | CO<sub>254</sub>   | 21 25 32 36 42 23 29 37 43      |                   |                        |
|                          | RFE<sub>254</sub>  | 21 25 32 36 42 18 23 29 37 43   |                   |                        |

**TABLE VIII**

Characteristic temperatures of mitochondrial membranes and extracted lipids from LM cells supplemented with choline analogues

Characteristic temperatures were obtained with ANS as in Table VII.

| Supplement               | Spectral parameter | Characteristic temperatures | Mitochondria | Mitochondria lipids |
|--------------------------|--------------------|-----------------------------|--------------|---------------------|
|                          |                    |                             |              |                     |
| Choline                  | CO<sub>254</sub>   | 19° 23° 33° 37° 45° 19° 25° 30° 37° 45° |              |                     |
|                          | RFE<sub>254</sub>  | 20 23 33 36 40 20 25 30 37 44   |              |                     |
| N,N'-Dimethylethanolamine| CO<sub>254</sub>   | 19 23 33 37 42 18 22 30 37 41   |              |                     |
|                          | RFE<sub>254</sub>  | 19 23 33 37 43 19 24 30 35 41   |              |                     |
| N-Monomethylethanolamine | CO<sub>254</sub>   | 18 23 32 37 42 18 25 31 36 43   |              |                     |
|                          | RFE<sub>254</sub>  | 19 22 33 37 41 20 24 30 36 44   |              |                     |
| Ethanolamine             | CO<sub>254</sub>   | 18 23 30 36 42 18 24 29 35 41   |              |                     |
|                          | RFE<sub>254</sub>  | 18 23 30 36 42 18 24 29 35 41   |              |                     |

**Fig. 3.** Values of CO and RFE of β-parinaric acid in plasma membranes or extracted lipids. CO<sub>213</sub> (open symbols) and RFE<sub>213</sub> (closed symbols) were determined at 15, 25, and 40° with β-parinaric acid (0.138 μg/ml) incorporated into plasma membranes or lipids as described under "Materials and Methods." Emission was measured at 415 nm.

Calculations were that the fatty acid composition of the phospholipids and other lipid fractions were essentially unchanged. The data previously presented appear to be consistent with this assumption. As shown in Table IX, the differences in the T<sub>r</sub> theoretical transition temperature, values calculated from LM phospholipid data were less than 5°, e.g., choline-supplemented cells had a T<sub>r</sub> about 5° lower than ethanolamine-supplemented cells. The figures in parenthesis indicate a difference of 11° if only the percentage of phosphatidylcholine had varied and phosphatidylethanolamine had remained constant. Similar considerations apply to the isolated membranes. Thus, by lowering both the percent-

As shown in Figs. 3 to 7, the values of the fluorescence parameters of β-parinaric acid and ANS varied in complex fashion with the degree of polar head group methylation of the base supplement. The observed changes varied for both probes depending upon the type of membrane in which they were incorporated. The weight of the experimental evidence suggests that the differences in the fluorescence behavior between the two probes may be due to a difference in the depth of penetration into the hydrophobic interior of the intact membrane as opposed to the isolated lipids. As shown above, ANS exhibits a large decrease in RFE when incorporated in the isolated lipid system, while β-parinaric acid shows a slight increase. This strongly suggests that ANS is more sensitive to surface effects on the isolated vesicles. This observation is not in disagreement with considerations of the distance between the charged group (located at the surface) and the fluorophore (located in the interior of the membrane) of the probe molecule.

Fluorescence parameters can be affected by a large variety of factors (10, 11, 23, 31, 44-50), so that interpretations of the absolute values of such quantities as corrected fluorescence or relative fluorescence efficiency in altered membranes are rendered very difficult. It has been shown that membrane proteins (51), surface charge of membranes (52-54), and even the presence of lysophosphatidylcholine-sterol complexes (55) can affect probe properties such as affinity or distribution. As demonstrated herein, the absolute values of CO<sub>213</sub> and RFE<sub>213</sub> incorporated into each membrane lipid varied with the degree
of polar head group methylation. Analogue supplementation decreased the corrected fluorescence of plasma membranes and plasma membrane lipids but increased the values of this parameter in microsomes, mitochondria, and their lipids. It is therefore possible that the specific lipid composition of each membrane and any alterations therein, rather than the presence of new phospholipids, may determine the absolute values of fluorescence probe spectral parameters. However, the various contributions of altered lipid composition to the fluorescence parameters are unknown at this time.

The probes ANS and \(\beta\)-parinaric acid are both negatively charged and would be expected to bind lipids containing quaternary ammonium groups much more readily than negatively charged lipids. It was previously shown that base analogues lowered the phosphatidylcholine content of mitochondria and plasma membranes more than in microsomes.\(^7\) This may lead to lowered affinity of the probes for the lipids. However, the results presented herein with ANS and mitochondria and previously with \(\beta\)-parinaric acid and plasma membranes\(^7\) indicate that analogue substitution decreased equilibrium dissociation constants (\(K_D\) values) with either the intact membranes or the isolated lipids. This can be interpreted as increased affinity of the lipids for the probes. Large decreases in affinity of probe for membrane or lipid were not noted. In addition the minimum number of phospholipid molecules providing a binding site for the probe was increased. Increased affinity for probe would be expected to increase fluorescence efficiency due to increased binding. However, increased size of binding site necessary for binding the probe would be expected to decrease the amount of bound probe and thereby decrease fluorescence. These two effects would therefore be opposed and would be complicated by the size of the bound water shell at the membrane surface. The complex effects of analogue substitution in LM membranes or the absolute values of the fluorescence parameters shown in Figs. 3 to 5 cannot be explained simply. It is possible that in addition to the binding effects the probes may interact differently with

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**Fig. 4.** Values of CO and RFE of \(\beta\)-parinaric acid in microsomes or extracted lipids. All methods were as in legend of Fig. 3.

**Fig. 5.** Values of CO and RFE of \(\beta\)-parinaric acid in mitochondria or extracted lipids. All methods were as in legend of Fig. 3.

**Fig. 6.** Values of CO and RFE of ANS in plasma membranes or extracted lipids. CO\(_{364}\) (open symbols) and RFE\(_{364}\) (closed symbols) were determined at 15, 25, and 40°C with ANS (4.16 \(\mu\)M) incorporated into plasma membranes or extracted lipids as described under "Materials and Methods." Emission was measured at 480 nm.

**Fig. 7.** Values of CO and RFE of ANS in mitochondria or extracted lipids. All methods were as in legend of Fig. 6.
Physical Properties of Membranes with Altered Phospholipids

Theoretical transition temperature $T_T$ of phospholipids from cells and subcellular fractions of LM cells grown in suspension culture with medium containing analogues of choline

| Cell fraction | Supplement | Sum of phosphatidyl-X (%) of total phospholipids | $T_T$ calculated from sum of diC16, phosphatidyl-X (%) |
|---------------|------------|-----------------------------------------------|-----------------------------------------------------|
| Whole cells   | Choline    | 76                                            | 48.8 (40.6)                                          |
|               | N,N'-Dimethylethanolamine | 76                     | 49.7 (46.5)                                          |
|               | N-Monomethylethanolamine | 69                     | 50.7 (41.6)                                          |
|               | Ethanolamine | 69                     | 53.0                                                  |
| Plasma membrane | Choline    | 67.4                                          | 50.5 (40.6)                                          |
|               | N,N'-Dimethylethanolamine | 64.1                   | 51.6 (48.3)                                          |
|               | N-Monomethylethanolamine | 58.4                   | 55.1 (52.9)                                          |
|               | Ethanolamine | 55.8                                          | 56.7                                                  |
| Microsomes    | Choline    | 60.7                                          | 54.6 (40.6)                                          |
|               | N,N'-Dimethylethanolamine | 57.1                   | 53.7 (46.4)                                          |
|               | N-Monomethylethanolamine | 55.8                   | 56.7 (54.2)                                          |
|               | Ethanolamine | 52.7                                          | 58.6                                                  |
| Mitochondria  | Choline    | 78.9                                          | 47.6 (40.6)                                          |
|               | N,N'-Dimethylethanolamine | 77.3                   | 49.5 (46.4)                                          |
|               | N-Monomethylethanolamine | 73.0                   | 53.7 (61.4)                                          |
|               | Ethanolamine | 71.7                                          | 59.8                                                  |

* X is choline, N,N'-dimethylethanolamine, N-monomethylethanolamine, and ethanolamine.
* Figures in parentheses indicate calculated $T_T$ from diC16, phosphatidyl-X where X is choline, N,N'-dimethylethanolamine, and N-monomethylethanolamine only (7, 8, 11).

Table IX

Each particular combination of lipids and that the dielectric strength, dipole-dipole interaction, or dipole-induced dipole interactions of the probe with its environment may drastically alter its quantum yield (10). Such effects have been shown to be important in polyene probes whose fluorescence parameters are responsive to polarizability but apparently not to solvent dielectric constant (11, 96). It is apparent from Figs. 3 to 5 that the effects of base analogues on fluorescence parameters are due to the lipids and possibly the protein may have an effect as well.

ANS has been shown to bind to the polar head group-hydrocarbon interface region of phospholipid micelles (54). The location of the probe, $\beta$-parinaric acid, is not known but it seems reasonable that like other fatty acids it would be expected to orient with the carboxyl group at the polar interface of lipid bilayers or membranes and with the alky chain embedded in the nonpolar core. The data indicated that $\beta$-parinaric acid interacted better with isolated lipids than with intact membranes. However, ANS bound only about half as well with the extracted lipids. Thus, although it has been shown that ANS can bind almost exclusively to lipids (90), ANS appears to interact with proteins as well as lipids in LM cell membranes. Both probes, which appear to interact with somewhat different regions of bilayers or membranes, reported very similar characteristic temperatures. Thus the characteristic temperatures reported by $\beta$-parinaric acid are not an exclusive property of that probe. Both ANS and $\beta$-parinaric acid indicated that base analogue incorporation into LM suspension cell membrane phospholipids had no effect on the characteristic temperatures. This would be consistent with the hypothesis that compensatory changes in lipid composition occurred to maintain a constancy of the characteristic temperatures of the lipid and membranes.

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