TABLE I
ROTATIONAL MOTION OF PROTEIN AND LIPID IN CYTOCHROME *c* REDUCTASE AND OXIDASE

Enzyme	τ_2 soluble (*s*)	τ_2 membrane (*s*)	Boundary lipid
Cytochrome oxidase: immobile	$\geq 1 \times 10^{-3}$	$\geq 1 \times 10^{-3}$	Immobile
Cytochrome oxidase: mobile	1×10^{-7}	4×10^{-5}	Mobile
Cytochrome reductase	4×10^{-7}	7×10^{-5}	Mobile

cation (1) at a lipid:protein ratio of 12.5:1 (by weight), results in a V_2' spectrum characteristic of a rotationally mobile membrane protein ($\tau_2 \simeq 7 \times 10^{-5}$s).

Because cytochrome reductase was obtained only as a rotationally mobile, and presumably disaggregated, protein, it was of interest to investigate whether lipid adjacent to the protein was mobile or not. To perform this experiment, we employed the MSL(1, 14) spin probe in which the nitroxide group is attached at one end of the acyl chain and the other end reacts with sulfhydryl groups through an *N*-ethylmaleimide linkage (5). MSL(1, 14) was added at a molar ratio of 2:1 to cytochrome reductase and noncovalently bound probe eliminated as previously described (1). As Table I also indicates, the hydrophobic environment adjacent to the protein was fluid, although an immobile component was also usually observed.

Our results demonstrate that mobile boundary lipid populations are detected by EPR when the protein moiety of either the reductase or oxidase complexes is rotationally mobile. Of course, a difficulty with these experiments arises from the fact that we do not know whether the protein environment is being uniformly sampled by the MSL(1, 14) probe.

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FATTY ACID PERTURBATION OF A MEMBRANE PROTEIN-LIPID INTERACTION

A TERBIUM (Tb^{3+}) FLUORESCENCE STUDY

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Recent studies suggest that immiscible lipid domains are an important structural feature of plasma membranes (1). By preferentially partitioning into a particular domain, free fatty acids (FFA) were found to alter lipid structure differentially, depending upon whether they are *cis* or saturated. These results, together with observed differential effects of FFA on membrane function (2, 3) suggest that FFA may, by means of protein-lipid interactions, alter the structure of proteins in particular domains. To test this hypothesis we chose to use Tb^{3+} fluorescence as a means of monitoring protein structural alteration as a function of FFA exposure. The rationale for using Tb^{3+} relies on the following: (*a*) significant Tb^{3+} fluorescence occurs only by energy transfer from a donor fluorophore, which from the observed excitation spectrum was shown in our case to be tryptophan; (*b*) Tb^{3+} has been shown to substitute for Ca^{2+} in many calcium binding proteins; and (*c*) it has been suggested that a calcium binding protein in lymphocyte plasma membranes is perturbed by exposure to *cis* but not saturated FFA (2).

RESULTS AND DISCUSSION

In this study plasma membranes were isolated from the CH1 mouse lymphoma line essentially by the method of Lemonnier et al. (5). Flourescence intensities were measured using a Perkin Elmer MPF2A fluorometer (Perkin-Elmer Corp., Instrument Div., Norwalk, CT), and lifetimes and polarizations were measured with a SLM 4800 instrument. Titration of the membranes with $TbCl_3$ resulted in saturation of the characteristic Tb^{3+} fluorescence (emission at 490 and 543 nm and excitation at 290 nm) at between 1 mol phospholipid: 0.75–1.0 mol $TbCl_3$. Incubation of the isolated plasma membranes with pronase had no effect on the saturation level or intensity of the Tb^{3+} fluorescence. Fluorescence was not observed when fresh whole cells were exposed to $TbCl_3$, suggesting that the site at which Tb^{3+} can fluoresce is on the cytoplasmic face of the cell plasma membrane. No essential difference was observed in the fluorescence polarization and lifetime of diphenylhexatriene (DPH) in control and Tb^{3+}-exposed membranes, suggesting that Tb^{3+} did not significantly alter membrane structure in the acyl chain region.

Tb-free membranes were incubated with ^{14}C-labeled *cis* (oleic and linoleic) and saturated (palmitic and stearic) FFA for 30 min at 22°C, with a FFA concentration of 25 mol% (relative to membrane phospholipid). Incorporation of FFA in membranes twice washed by centrifugation was found to be virtually 100%. Exposure to FFA had no effect on the protein:lipid ratio, 1.25:1 (wt/wt), indicating little or no solubilization by FFA. However, as shown by DPH polarization studies, FFA can alter membrane lipid structure (1, 6). To monitor the effect of free fatty acid on Tb^{3+} fluorescence, control and FFA-exposed membranes were tritrated with $TbCl_3$. As shown in Fig. 1, the intensity, relative to the control, was ~35% greater in membranes treated with stearic acid and relatively unchanged in those treated with *cis* FFA. The amount of binding of the radio isotope $^{160}Tb^{3+}$ was found to be unaffected by FFA exposure, assuring that the observed changes in fluorescence were not due to changes in the amount of membrane-bound Tb^{3+}.

To interpret these findings in terms of a FFA-induced conformational change in a membrane protein(s) it is necessary to determine whether the fluorescence represents Tb^{3+} bound to specific protein sites. The alternative is that the fluorescence is due to transfer from membrane tryptophan to phospholipid-associated Tb^{3+}. Although the increased energy transfer is not the result of an increase in total Tb^{3+} binding to the membranes, it could be due to a redistribution of lipid-bound Tb^{3+}. However, the amount of energy transfer expected from nonspecifically located Tb^{3+} is negligible. Because the mean separation between lipid molecules is > 8 Å and the Tb^{3+} binding site on the phospholipid is probably in the vicinity of headgroup phosphorous, it is likely that nonspecifically bound Tb^{3+} is 10 Å from a tryptophan residue. To evaluate the importance of transfer from these nonspecifically bound Tb^{3+}, we calculated the two-dimensional Förster transfer rate using R_o = 3.5Å. These calculations demonstrate that at a surface density of 0.25 Tb^{3+}/phospholipid the energy transfer efficiency from a single tryptophan is < 0.09%. Since the range of the transfer mechanism is probably shorter than the Förster mechanism and the pronase experiments suggest that tryptophan is buried, this estimate represents an upper limit. Conversely, the energy transfer efficiency actually obtained from the Tb^{3+}-sensitized emission at a surface density of 0.25 Tb^{3+}/phospholipid was > 0.5%. This, together with the fact that labeling of whole cells does not elicit Tb^{3+} fluorescence, suggests that the fluorescent Tb binding site is a specific protein(s) accessible only from the cytoplasmic side of the membrane.

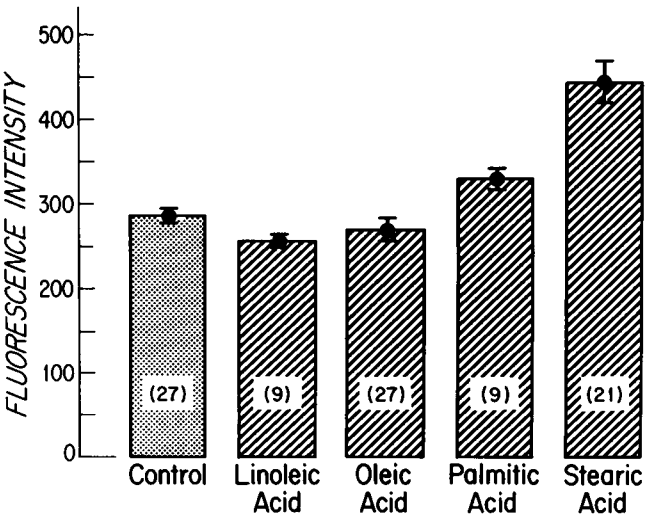

FIGURE 1 Tb^{3+} fluorescence in CH1 membranes. Errors are standard error of the mean, numbers in parentheses represent sample size. SA intensities are significantly greater than control, *t* test > 99%. Buffer: 10 mM Tris (pH 7.4), 120 mM NaCl, 0.2% NaN_3.

It is possible that the alteration of Tb^{3+} fluorescence is due to direct protein-fatty acid interactions. Differential effects we observe would then imply that the protein-fatty acid interaction is sensitive to the physical state of the fatty acid acyl chain. Although changes in DPH polarization suggest that most FFA are incorporated into the lipid phase, in the present experiments we cannot exclude the possibility that a small fraction of the FFA binds directly to the protein(s), causing conformational change. Nevertheless, we conclude that the observed changes in Tb^{3+} fluorescence are a result of conformational alterations in what are probably Ca^{2+}-binding membrane proteins.

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INTERACTION OF PHOSPHATIDYLCHOLINE WITH β-LACTOGLOBULIN

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Although β-lactoglobulin (β-Lg), the major whey protein of bovine milk, has no known membrane-associated function, it shares certain structural characteristics with membrane proteins. Both average hydrophobicity and the ratio of charged to hydrophobic residues lie between those for intrinsic and extrinsic membrane proteins (1). The circular dichroism (CD) spectrum of native β-Lg shows much less helical structure than predicted by analysis of the amino acid sequence by use of the Chou-Fasman rules (2). Segrest and Feldman (3) identified residues 130–143 of its sequence as a possible amphipathic helix similar to those found in lipoproteins and some membrane proteins. When β-Lg is synthesized in vitro in the presence of microsomes, its signal peptide is cleaved while the protein is passing through the membrane, and β-Lg then assumes its native conformation (4). In a microsome-free system, the signal peptide is not cleaved and is not accessible to enzymatic attack after the protein folds. These observations led us to look for possible interactions of β-Lg with phospholipids. Although no complex formation has been detected between native β-Lg and egg phosphatidylcholine, dimyristoyl phosphatidylcholine (DMPC), or dipalmitoyl phosphatidylcholine (DPPC), solvent-denatured β-Lg readily interacts with each of these lipids.

MATERIALS AND METHODS

Dry lipid was added to β-Lg in a single-phase system formed by diluting 10 mg/ml β-Lg in 0.14M KCl with 2:1 $CHCl_3$:CH_3OH and acidifying with 3% HCl in CH_3OH.[1] Solvents were removed under N_2 and the resulting film was dispersed in buffer. Vesicles formed by the method of Barenholz et al. (5) provided a system suitable for optical and resonance spectroscopy, allowing observation of the effect of lipid on protein and protein on lipid. Measurements at pH 3.7 and pH 7.2 allowed us to observe the effects of changes in the charge distribution on β-Lg. CD spectra were obtained with a J-41C spectropolarimeter (Jasco Inc., Easton, MD)[2], ^{31}P NMR with an FX 60Q spectrometer (JEOL, USA, Cranford, NJ) and electron micrographs with a 10-B electron microscope (Carl Zeiss, New York, NY).

RESULTS AND DISCUSSION

At pH 3.7, DPPC vesicle suspensions were unstable and quickly became turbid. Vesicles formed from the β-Lg-DPPC complex were more stable. The ^{31}P NMR spectrum consisted of a narrow line, indicating rapid motion of the head groups. Electron micrographs of these vesicles showed them to be uniformly dispersed but somewhat flattened, with the short diameter approximately one-third that of the long diameter. These dispersions were suitable for ultraviolet (UV) absorption spectroscopy. The far UV CD spectrum (Fig.1 *a*) shows native β-Lg to have predominately β-structure. Our solvent system is helix forming, but the CD spectrum of solvent-treated, redissolved β-Lg without lipid shows only β- and unordered structures. The double minimum in the spectrum of the β-LG-DPPC vesicle complex represents 200% of the helix in the native protein. The near UV CD spectra (Fig. 1 *b*) of native and solvent-treated β-Lg are similar and distinctly different from the spectrum of the vesicle complex.

At pH 7.2, DPPC vesicle suspensions were stable, but the inclusion of denatured β-Lg led to pelleting of both lipid and protein when the suspension was centrifuged. Electron micrographs of the DPPC vesicles showed a

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[1]Brown, E. M., J. Sampugna, R. J. Carroll, and P. E. Pfeffer. Submitted for publication.

[2]Reference to brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.