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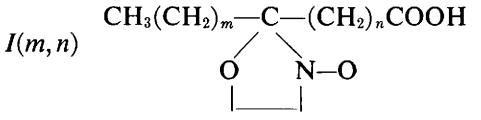
Spin Label Studies on Rat Liver Plasma Membrane: Calcium Effects on Membrane Fluidity (38967)

RICHARD D. SAUERHEBER AND LARRY M. GORDON
(Introduced by A. N. WICK)

Dept. of Chemistry, San Diego State University, San Diego, CA 92182; and the California Metabolic Research Foundation, La Jolla, CA 92037

Ca^{2+} participates in a number of membrane-associated physiological functions, including regulation of membrane-bound enzymatic activities, release of secretory products, maintenance of cell shape, functioning of transport systems, neuronal conduction, and muscle contraction (1, 2). Rasmussen has recently reviewed the relationship. between Ca^{2+} and cyclic AMP as second messengers for certain hormones (3, 4). Ca^{2+} release from the plasma membrane has been proposed to play an important role in the action of insulin (5). Furthermore, Shlatz and Marinetti (6) have found that various hormones exert pronounced effects on Ca^{2+} binding to rat liver plasma membrane.

The effects of various ions on the properties of model and biological membranes have been widely examined. Ca^{2+} alters the permeability, excitability, and adhesiveness of these structures (1, 7). Furthermore, Ca^{2+} has been shown to increase the rigidity of model membranes, as monitored with spin-labeled cholestane (8) and of membranes from *Bacillus subtilis*, labeled with nitroxide derivatives of stearic acid I(m, n) (9).



$I(m, n)$ have been used to obtain information relative to membrane fluidity in both artificial and natural membrane systems (10–12). An order parameter S, which is sensitive to membrane fluidity, can be derived from ESR spectra of labeled membranes (13, 14).

Ca^{2+} effects on the fluidity of rat liver plasma membranes have not been examined with $I(m, n)$ probes. In this paper we report the preparation of rat liver plasma membrane using a Sorvall SZ-14 reorienting density gradient zonal procedure in which the gradient and sample were dynamically loaded and statically unloaded. The ESR spectra obtained by incorporating spin-labeled derivatives of fatty acids into these membranes are also presented. Alterations in spectral parameters induced by temperature changes and addition of $CaCl_2$ are also reported.

Materials and Methods. Chemicals. Spin-labeled stearic acid derivatives, 5-nitroxide stearate, I (12, 3), and 16-nitroxide stearate, I (1, 14), where nitroxide refers to the 4′,4′-dimethyloxazolidine-*N*-oxyl group, were purchased from Syva Co., Palo Alto, CA 94304. All other chemicals were purchased from Sigma Chemical Company, St. Louis, MO.

Enzyme assays. 5′-Nucleotidase activity was measured according to the procedure of Michell and Hawthorne (15). Na^+, K^+-ATPase and Mg^{2+}-ATPase were assayed according to Bonting and Caravaggio (16). Inorganic phosphate released in the above assays was measured according to King (17). Monoamine oxidase (MAO) activity was determined by the procedure described by Tabor *et al.* (18). Protein was determined by the biuret method.

Preparation of plasma membranes. Rat liver plasma membranes were prepared by modifications of the procedure described by Evans (19). The resuspended low speed (crude nuclear) fraction from 40 g of Sprague–Dawley rat liver was dynamically loaded into a Sorvall SZ-14 reorienting density gradient zonal rotor which had been previously loaded with the following prechilled sucrose solutions containing 1 m*M* $NaHCO_3$ (pH 7.6): a cushion of 85 ml 54% (w/v), a linear gradient of 300 ml of 54% mixed with 300 ml of 36%, a linear gradient of 100 ml of 36% mixed with 100 ml of 24%, 100 ml of 24%, and 300 ml of 6%. After loading the sample, a 20 ml overlay of 1 m*M* $NaHCO_3$ (pH 7.6) was added, thereby closely reproducing the conditions of Evans (19).

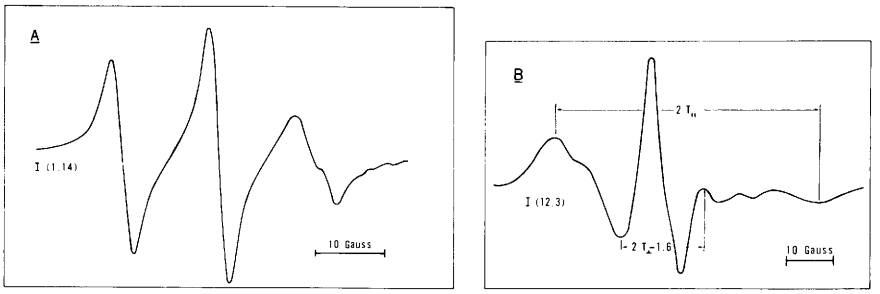


FIG. 1. ESR spectra of nitroxide-labeled stearic acid in solution with an aqueous dispersion of liver plasma membrane. A: spectrum of I (1, 14). B: spectrum of I (3, 12). S is calculated (13, 14, 29) from the spectra of I (12, 3) from the expression:

$$S = (T_{\parallel} - T_{\perp})/(T_{zz} - T_{xx}),$$

where T_{zz} and T_{xx} are the values of the hyperfine coupling tensor in the z and x directions (assuming axial symmetry) from spectra of nitroxide containing single crystals, and $T_{\parallel}$ and $T_{\perp}$ are measured as shown. $2T_{\perp}$ is corrected by the addition of 1.6 Gauss, the approximate value of two half-widths of the inner hyperfine lines (29).

The rotor was spun at 3900 rpm for 50 min and then decelerated (using the Sorvall rate controller). The gradient and contents were statically unloaded into 15-ml fractions. Each of these fractions was diluted to 20 ml with 1 mM $NaHCO_3$ (pH 7.6) and centrifuged at 40,000g for 20 min. The pellets were resuspended in 8% sucrose, 5 mM Tris–HCl (pH 7.6). Aliquots were assayed for enzyme activity and protein; separate aliquots were examined with a Zeiss phase contrast microscope.

Spin labeling. Spin probes were dissolved in ethanol (10^{-3} M) and 7.5 μl aliquots were dried under reduced pressure in glass centrifuge tubes. Aliquots of plasma membrane were then added, followed by gentle vortexing at room temperature for several minutes. Spin labels were incorporated at a ratio of 1 per 140 lipid molecules (assuming all membrane lipid to be phospholipid with an average MW of 780 g/mole with 1 mg of phospholipid/mg of membrane protein (19). ESR spectra were recorded using a small aqueous cell (0.15 ml capacity) with a Varian E-3 ESR spectrometer equipped with a variable temperature accessory.

Results. The sedimentation profile of the membrane homogenate is similar to that previously reported by Evans (19). The highest specific activity of 5′-nucleotidase occurred in the 49% sucrose portion of the gradient and is attributed to plasma membrane fragments (19, 20). The presence of membrane fragments was confirmed by examination with phase contrast microscopy. One of the membrane fractions was assayed for Na^+, K^+-ATPase and Mg^{2+}-ATPase activities prior to pooling on the basis of the 5′-nucleotidase activity. The specific activities were 0.35 and 1.53 μmoles phosphate released/hr/mg protein, respectively. Pooled membrane was judged to be essentially free of mitochondria because: (1) The preparation contained no detectable MAO activity and (2) Examination by phase contrast microscopy indicated minimal mitochondrial contamination in the preparation.

The spectra of membranes containing I (1, 14) and I (12, 3) at room temperature are shown in Fig. 1A and B. The nitroxide in the 5 carbon position of stearic acid appears to be more immobilized than the 16 carbon derivative. Membrane aliquots (0.5–0.25 ml) were titrated with the probe to maximize the signal to noise ratio, while minimizing the concentration of probe in the membrane.

The order parameter S (13, 14) was 0.656 at room temperature in the liver plasma membrane preparation. S was determined experimentally from the spectra of I (12, 3) as shown in Fig. 1B. A plot of $(1 - S)/S$ versus temperature (Fig. 2) is biphasic, with an apparent transition at 20°. These results are similar to those found in a study employing kidney plasma membrane (21).

Ca^{2+} effects on the order parameter of I (12, 3) at 37° are presented in Fig. 3. ΔS, the percent change in S, was measured for 0.65, 1.96, 3.80, and 7.00 mM $CaCl_2$ additions. Positive increases in ΔS occurred with incubation of the membranes with 0.61 and

1.96 mM $CaCl_2$. However, no further increase in ΔS occurred with $CaCl_2$ concentrations exceeding 1.96 mM. $CaCl_2$ was incubated with the membrane for at least 10 min at 37° (22) before the ESR spectra were recorded.

Discussion. Zonal centrifugation has been used for the isolation of many subcellular fractions (23). Several accounts have emphasized the advantages of this method for large-scale preparations of plasma membrane (19, 24–27), and no difficulties were encountered using the reorienting zonal rotor for this study.

The evidence that spin labels were incorporated into an anisotropic bilayer structure is (1) The spectra of I (1, 14) are much more isotropic than those of I (12, 3) and (2) Probed membranes which were centrifuged (40,000g for 20 min) and resuspended in fresh buffer yielded minimal loss of signal. The order parameter dependence on temperature of I (12, 3) indicates liver plasma membrane lipids might undergo a phase change (or a phase separation) at 20° similar to other natural and artificial membrane systems (21).

Our results show that Ca^{2+} decreased the fluidity of liver plasma membrane. Fluorescence studies on model systems (28) and ESR work on *Bacillus subtilis* membrane (9) have also indicated Ca^{2+} increased the membrane's rigidity.

$^{45}Ca^{2+}$ binding studies with isolated liver plasma membranes indicated two distinct binding sites: a high affinity site (association

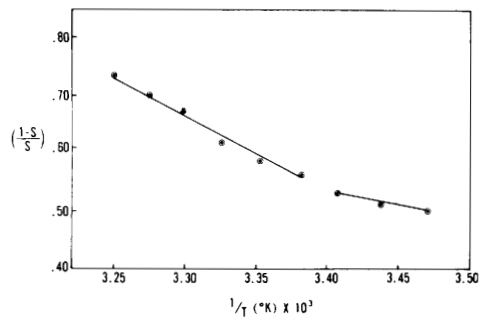


FIG. 2. Temperature dependence of $(1\text{-}S)/S$, S calculated from the spectra of I(12, 3) in liver plasma membrane as indicated in Fig. 1B. The temperature range was 15–35°.

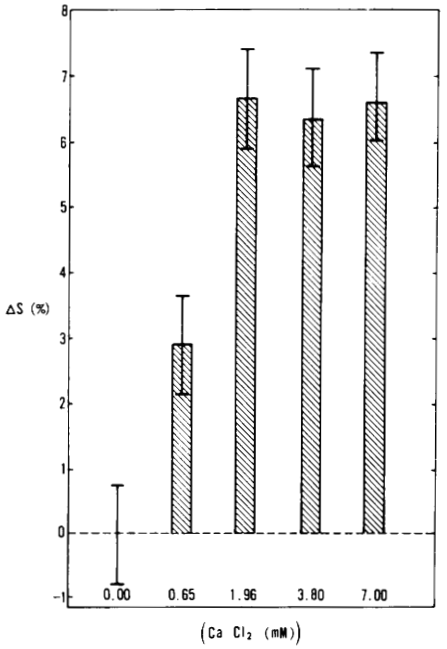


FIG. 3. The effects on S of additions of various concentrations of $CaCl_2$ to the spin-labeled membranes. The error bars indicate ± 1 SD.

constant, $K_a = 4.0 \times 10^3\ M^{-1}$), and a low affinity site ($K_a = 3.2 \times 10^2\ M^{-1}$). These Ca^{2+} binding sites were described as ionized anionic functional groups on the membrane (carboxyl and phosphate groups) (23). The preliminary studies reported here indicate the membrane is saturated with Ca^{2+} at 1.96 mM, since further addition of Ca^{2+} failed to increase ΔS. This is in agreement with the $^{45}Ca^{2+}$ study which showed saturation of the low affinity sites at similar Ca^{2+} concentrations. Further study of ΔS at lower concentrations of $CaCl_2$ to probe high and low affinity sites is clearly warranted.

Since several hormones acting on the liver have been reported to influence Ca^{2+} binding to liver plasma membrane (6), it is tempting to suggest these hormones could regulate various effector systems (e.g., membrane-bound enzymatic activity, ion transport, etc.) by altering the fluidity of hydrophobic regions of the membrane. Lipids are known to play an important role in the expression of various membrane-bound activities (21, 29). Moreover, lipid phase transitions have been correlated with certain membrane-bound en-

zymatic activity transitions in Arhennius plots (21, 29). Recent studies by McConnell (30, 31) indicate that epinephrine, prostaglandins, and various neurotransmitters cause detectable fluidity changes in red blood cells in the presence of Ca^{2+}. Since hormone effects on Ca^{2+} binding occur at physiological concentrations, we suggest that monitoring membrane fluidity might provide a feasible system for studying hormonal regulation in liver plasma membrane.

Summary. Spin-labeled stearic acid probes were incorporated into rat liver plasma membrane purified by a zonal centrifugation procedure. Temperature effects on the order parameter calculated from the spectra indicated the possible presence of a membrane phase transition at 20°, in agreement with earlier studies in other systems. Addition of Ca^{2+} (1.96 m*M*) at 37° increased the order parameter by 6.0 ± 0.7%, suggesting a Ca^{2+}-mediated decrease in membrane fluidity.

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