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Effects of Calcium, Lanthanum, and Temperature on the Fluidity of Spin-Labeled Human Platelets

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Summary. Previous platelet studies have shown that calcium plays important roles in stimulus-secretion coupling, aggregation, and other membrane-associated functions. In addition, lanthanum induces platelet aggregation and the platelet release reaction and also influences platelet responsiveness to various stimuli. The spin-label results presented here suggest that one mechanism through which calcium and lanthanum mediate their effects on platelet functions may be by decreasing the lipid fluidity of the surface membrane.

The structure of platelet membrane lipids was examined with the spin-label method. Washed human platelets were labeled with the 5-, 12- and 16-nitroxide stearic acid spin probes. Order parameters which measure the fluidity of the lipid environment of the incorporated probe may be calculated from the electron spin resonance (ESR) spectra of 5-nitroxide stearate [I(12,3)]-labeled cells. Evidence is presented which indicates that these spectra principally reflect properties of the platelet surface membrane lipids. The membrane fluidity increased with temperature for the range 17 to 37 °C. Either calcium or lanthanum additions to intact cells increased the rigidity of the platelet membranes at 37 °C, although the La^{3+} effect was larger and occurred at lower concentrations than that of Ca^{2+}. For example, addition of 1 mM La^{3+} or 4 mM Ca^{2+} increased the order parameter of I(12,3)-labeled platelets by $4.3 \pm 1.7\%$ or $2.1 \pm 0.5\%$. Preliminary studies conducted on purified platelet plasma membranes labeled with I(12,3) indicated that 1 mM $LaCl_3$ or 4 mM $CaCl_2$ additions similarly decreased the lipid fluidity at 37 °C. The above cation-induced effects on the fluidity of whole platelets were reversed by the use of the divalent cation-chelating agent ethylene glycol-bis-(β-aminoethyl ether)-N,N′-tetra-acetic acid (EGTA). Lastly, lanthanum (0.2–1 mM) caused rapid aggregation of platelets which were suspended in a 50-mM Tris buffer pH 7.4 that did not contain adenosine.

The importance of calcium in regulating a variety of platelet physiological processes is well known. Certain studies indicate that the platelet "shape change" from a smooth discoid to a spiny spherical morphology, the release reaction, and the intracellular metabolic activity of stimulated platelets may be controlled by the availability of calcium within the cytoplasm (Lüscher & Massini, 1975; Schneider & Gear, 1975; Rasmussen & Goodman, 1977). Furthermore, extracellular calcium is required for platelet adhesion and aggregation to occur (Hellem, 1960; Cronberg & Caen, 1970; Heptinstall, 1976; Grinnel, 1978). Although conflicting evidence has been reported concerning the need for extracellular calcium to induce the platelet release reaction (Mustard & Packham, 1970), the level of calcium in the medium appears to modulate the intensity of the platelet response to release-inducing agents (Sneddon, 1972).

Attention has focused recently on the effects that calcium exerts on platelet surface membrane-associated functions. Calcium has generally been found to influence numerous membrane processes, such as the permeability, adhesiveness, and excitability of biological membranes (Manery, 1966; Watanabe & Tasaki, 1971; Rubin, 1974; Nicolson, Poste & Ji, 1977). In platelets, calcium modulates the activities of membrane-associated ATPase (Chambers, Salzman & Neri, 1967; Wang *et al.*, 1977) and adenylyl cyclase (Vigdahl, Marquis & Tavormina, 1969; Rodan & Feinstein, 1976). Moreover, the permeability of the platelet membrane to $^{45}Ca^{2+}$ appears to

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increase when platelets are treated with agents such as thrombin, ADP, and epinephrine (Lüscher & Massini, 1975; Massini & Lüscher, 1976; Robblee & Shepro, 1976). Stimulated platelets also uptake appreciable amounts of $^{45}Ca^{2+}$ from the medium; washing either activated or control platelets with La^{3+} releases a significant fraction of the accumulated $^{45}Ca^{2+}$, suggesting that a calcium pool may be associated with the cell surface (Robblee & Shepro, 1976). La^{3+} has commonly been employed as a probe of Ca^{2+}-plasma membrane binding sites since this cation not only exhibits a high binding affinity for such sites but also does not penetrate intact cells (Weiss, 1974; Mikkelsen, 1976; Robblee & Shepro, 1976). The above La^{3+} effects on $^{45}Ca^{2+}$ binding are of particular interest in view of the profound influence that La^{3+} has on platelet aggregation and the release reaction (Wörner & Brossmer, 1976).

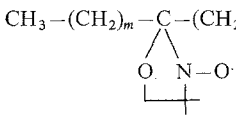
An adequate understanding of how Ca^{2+} and La^{3+} regulate various platelet membrane-associated functions requires the elucidation of the underlying structural alterations induced by these cations. Biological membranes are currently viewed as having "fluid" properties; endogenous membrane lipids and proteins each exhibit relatively free rotational and translational motions (Jain & White, 1977). Indeed, several investigators have suggested that an appropriately fluid membrane is required for normal cellular function. Lee (1975) and Jain and White (1977) have reviewed numerous membrane activities which might be affected by the fluidities of constituent lipid domains. It is therefore worthwhile to note that Ca^{2+} and La^{3+} have been found in previous physical-biochemical studies to decrease the lipid fluidity of both model and biological membranes (Butler, Tattrie & Smith, 1970; Ehrström *et al.*, 1973; Träuble & Eibl, 1974; Lee, 1975; Marsh, Radda & Ritchie, 1976; Uyesaka *et al.*, 1976; Breton, Viret & Leterrier, 1977; Nicolson *et al.*, 1977; Weller & Haug, 1977; Gordon, Sauerheber & Esgate, 1978). An important question that should be addressed concerns whether Ca^{2+} or La^{3+} similarly decrease the lipid fluidity of platelet surface membranes. Studies investigating this problem might be of singular interest, in view of the proposal by Cohen that the binding of Ca^{2+} to platelet surface membrane phospholipids may produce a lipid "compression wave" which could modify membrane functions (Cohen & De Vries, 1973; Lüscher & Massini, 1975).

The spin-label method was employed here as a tool to examine the effects that temperature alterations and Ca^{2+} and La^{3+} additions exert on the lipid structure of platelet surface membranes. The electron spin resonance (ESR) spectra of human platelets labeled with nitroxide derivatives of stearic acid were used to monitor the "fluidity" of the lipid environment of the probe. Experimental evidence is presented that suggests that the spin label primarily samples the surface membrane. Comparison of the fluidities of platelets with other biological membranes indicates that washed platelets exhibit a relatively rigid membrane lipid structure at 30 °C. Lastly, additions of mM Ca^{2+} or La^{3+} decreased the fluidities of labeled platelets at 37 °C; similar concentrations of La^{3+} also promoted platelet clumping under appropriate conditions.

Materials and Methods

Materials

The spin labels employed in this study were N-oxyl-4′,4′-dimethyloxazolidine derivatives of stearic acid, sharing the following general formula:



I(m, n)

The nitroxide derivatives of 5-ketostearic acid [I(12,3)],12-ketostearic acid [I(5,10)], and 16-ketostearic acid [I(1,14)] were purchased from Syva Co., Palo Alto, Calif. The I(m, n) labels were dissolved in absolute ethanol (at 10^{-3} M) and stored at −20 °C in liquid nitrogen storage tubes (Microbiological Associates).

All other chemicals were from Sigma Chemical Co., St. Louis, Mo. Stock solutions of $CaCl_2$, $LaCl_3$, EGTA, and $K_3Fe(CN)_6$ were made by dissolving these agents in either 50 mM Tris-HCl (pH 7.4) or distilled water.

Platelet Isolation

Blood was obtained from healthy donors and anticoagulated with citrate-phosphate dextrose (CPD). Platelet-rich plasma (PRP) was prepared by a 10-min centrifugation at $200 \times g$. The platelets were then collected by centrifuging the PRP two times at $800 \times g$ for 10 min. The cells were subsequently washed three times by repeated centrifugation at $500 \times g$ for 5–10 min in 50 mM Tris-HCl (pH 7.4) buffer, containing 100 mM NaCl and (unless otherwise indicated) 0.03 M adenosine. The few remaining red blood cells were removed by a final centrifugation step at $1{,}000 \times g$ for 15 sec. The platelets were usually suspended at a concentration of approximately 3×10^6 cells per µl. Plastic pipettes and containers were used for all platelet isolation steps.

Spin Labeling

Aliquots of the spin labels were first evaporated to dryness in liquid nitrogen storage tubes (Cole Scientific Co.) with dry N_2 gas. The platelet preparation was gently shaken, and 30–60 µl of the suspension was added to the probe. The total sample volume was adjusted to 60 µl by adding Tris-HCl buffer (pH 7.4) containing 100 mM NaCl and 0.03 M adenosine, when necessary. The platelet sample containing the spin probe was gently hand vortexed at room temperature (*RT*).

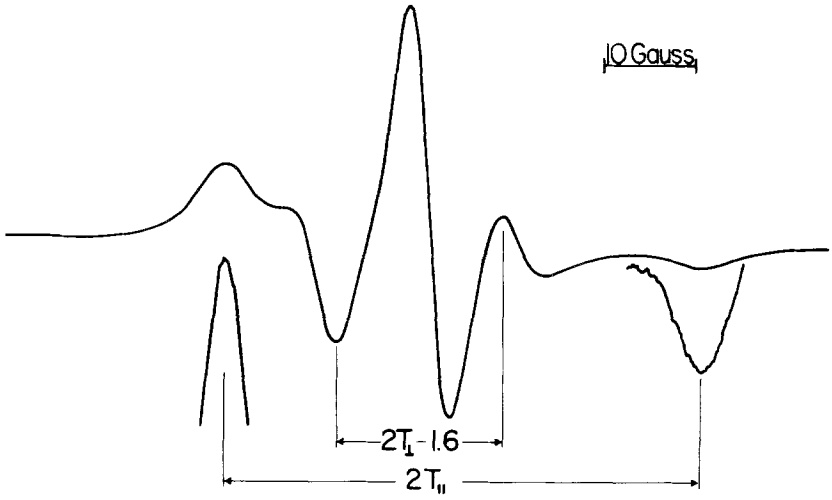


Fig. 1. ESR spectrum of I(12,3)-labeled and washed human platelets at 37 °C. The spectrum was recorded with a 4-min scan time, 5×10^4 receiver gain, and a 100-G field sweep. The outer peaks were also magnified by recording at 5×10^5 receiver gain and a 16-min scan time (*see* Materials and Methods). The outer and inner hyperfine splittings, $2T_{\|}$ and $2T_{\perp}$, were measured as shown; $2T_{\perp}$ was corrected by the addition of 1.6 G (Hubbell & McConnell, 1971). The probe/cell ratio was 4 μg I(12,3)/1.5×10^8 cells

The spin-labeled platelets were drawn into 50-μl, siliconized capillary pipettes (Drummond Scientific Co.) and then sealed at one end with Critoseal (Scientific Products). The spin label/platelet ratio employed was varied from approximately 4 to 33 μg I(*m*, *n*)/1.5×10^8 cells. The probe/cell ratio was calculated assuming the platelets quantitatively incorporated the spin label.

Spectra were recorded with a Varian E-104A Century Series ESR spectrometer equiped with a variable temperature accessory. Capillary pipettes containing the labeled samples were placed in a special holder [designed by R.D. Kornberg (Gaffney, 1974)] that was mounted in the temperature accessory. A thermocouple readout meter (Omega Engineering, Stamford, Conn.) with 0.01 mm diameter wires leading to the center of the cavity indicated the sample temperature throughout each experiment. Spectra were measured after a 4–5 min wait for temperature equilibration. The microwave power was kept at 10 mW which neither heated the platelet sample nor saturated the ESR signal.

Agent Additions to I(12,3)-Labeled Platelets at 37 °C

The effects of such agents as $CaCl_2$, $LaCl_3$, NaCl and EGTA on the fluidity of platelets were examined as follows. An ESR spectrum of the platelet sample labeled with a given I(12,3) probe concentration was initially recorded at 37 °C after a 4-min wait for temperature equilibration. An aliquot of a concentrated stock solution of the agent was then added to the platelet sample and the ESR spectrum measured after an 8-min wait at 37 °C. The above protocol was employed inasmuch as previous studies on platelets indicated that $^{45}Ca^{2+}$ accumulation from the extracellular medium attained a steady state within several minutes at *RT* (Robblee & Shepro, 1976). The reversibility of the effects of $CaCl_2$ or $LaCl_3$ was tested in several experiments by recording the spectrum of the platelet sample containing the cation, and then measuring the spectrum at 37 °C after adding the Ca^{2+}- (and La^{3+}-) chelating agent EGTA and waiting 8 min.

Spectral Measurements

Figure 1 shows the ESR spectrum of I(12,3)-labeled human platelets, recorded at 37 °C with a 100-G field sweep, 5×10^4 receiver gain and 4-min scan time. The spectrum indicates that the label undergoes rapid anisotropic motion about its long molecular axis in the membrane; flexing or bending motions of the probe (i.e., the angular deviation of the hydrocarbon chain away from the preferred orientation perpendicular to the membrane surface) appear to be relatively restricted. Similar spectra have been reported for other I(*m*, *n*)-labeled model and biological membranes (Hubbell & McConnell, 1971; Gordon & Sauerheber, 1977; Sauerheber *et al.*, 1977; Gordon *et al.*, 1978). The flexibility, or "fluidity", of the membrane-incorporated label may be quantitated by measuring the outer and inner hyperfine splittings $2T_{\|}$ and $2T_{\perp}$ in Fig. 1 The outer peaks were magnified with a tenfold higher gain setting and 16 min scan time; peak positions were determined as described previously (Gordon *et al.*, 1978). Each spectrum was recorded in approximately 8 min.

The following order parameter expressions (Gordon & Sauerheber, 1977) may be used to evaluate the flexibility of platelet membrane-incorporated fatty acid spin labels:

$$S(T_{\|}) = 1/2\left[\frac{3(T_{\|} - T_{xx})}{(T_{zz} - T_{xx})} - 1\right] \tag{1}$$

$$S(T_{\perp}) = 1/2\left[\frac{3[(T_{zz} + T_{xx}) - 2T_{\perp}]}{(T_{zz} - T_{xx})} - 1\right] \tag{2}$$

and

$$S = \left(\frac{T_{\|} - T_{\perp}}{T_{zz} - T_{xx}}\right)\left(\frac{a_N}{a'_N}\right). \tag{3}$$

Here, T_{xx} and T_{zz} are the hyperfine splitting elements of the static interaction tensor (**T**) parallel to the static Hamiltonian (**H**) principal nuclear hyperfine axes x and z, respectively. The x axis is parallel to the N-O bond direction, and the z axis is parallel to the nitrogen $2p$ π orbital. The elements of **T** used in this study were previously determined by incorporating nitroxide derivatives into host crystals as substitutional impurities: $(T_{xx}, T_{zz}) = (6.1, 32.4)$ G (Seelig, 1970). A'_N and a_N are the isotropic hyperfine coupling constants for the probe in the membrane and crystal state, respectively [i.e., $a'_N = 1/3(T_{\|} + 2T_{\perp})$ and $a_N = 1/3(T_{zz} + 2T_{xx})$].

The order parameters S, $S(T_{\|})$ and $S(T_{\perp})$ are sensitive to the membrane fluidity (or, more accurately, the flexibility of the membrane-incorporated probe). S, $S(T_{\|})$ and $S(T_{\perp})$ may each assume values between 0 and 1; these extreme order parameters indicate that the probe samples fluid and immobilized environments, respec-

tively. The order parameter S, which requires both hyperfine splittings, corrects for small polarity differences between the membrane and reference crystal. Although $S(T_{\parallel})$ and $S(T_{\perp})$ do not include corrections for polarity contributions, these expressions have been found to be useful approximate measures of the fluidity in those cases where only one hyperfine splitting is useable (Gordon & Sauerheber, 1977). Previous studies of I(12,3)-labeled biological membranes indicated that $2T_{\perp}$ increased with the probe concentration at high probe/lipid ratios, while $2T_{\parallel}$ remained virtually unchanged (Sauerheber *et al.*, 1977; Gordon *et al.*, 1978); accordingly, S and $S(T_{\perp})$ decreased, while $S(T_{\parallel})$ was constant with increasing probe concentration. We attributed these spectral changes to enhanced nitroxide radical interactions between probe molecules. Since similar probe-concentration dependent spectral alterations were observed in I(12,3)-labeled human platelets, $S(T_{\parallel})$ has primarily been employed in this paper to monitor the platelet membrane fluidity.

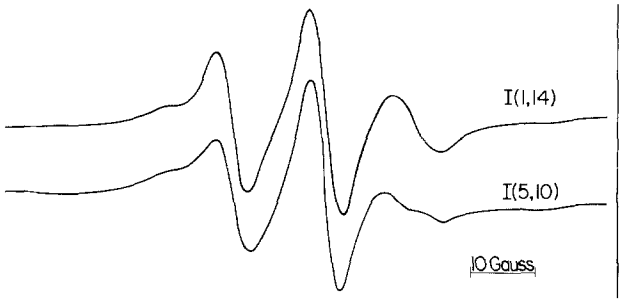


Fig. 2. ESR spectra of I(5,10)- and I(1,14)-labeled platelets. The spectra of platelets labeled with I(5,10) and I(1,14) were recorded at 37 °C at a 100-G field sweep, 5×10^4 receiver gain and 4 min scan time. The platelets were labeled with 10 μg I(m, n)/1.5×10^8 cells

Results

The Anisotropic Spectra of I(m, n)-Labeled Platelets

ESR spectra of washed human platelets labeled with the I(12,3), I(5,10) and I(1,14) spin probes at 37 °C are shown in Figs. 1 and 2. The asymmetric spectra indicate that each of these nitroxide labels exhibits rapid, enhanced rotation about their long hydrocarbon chains. However, the I(5,10) and I(1,14) spectra are considerably less anisotropic than the I(12,3) spec-

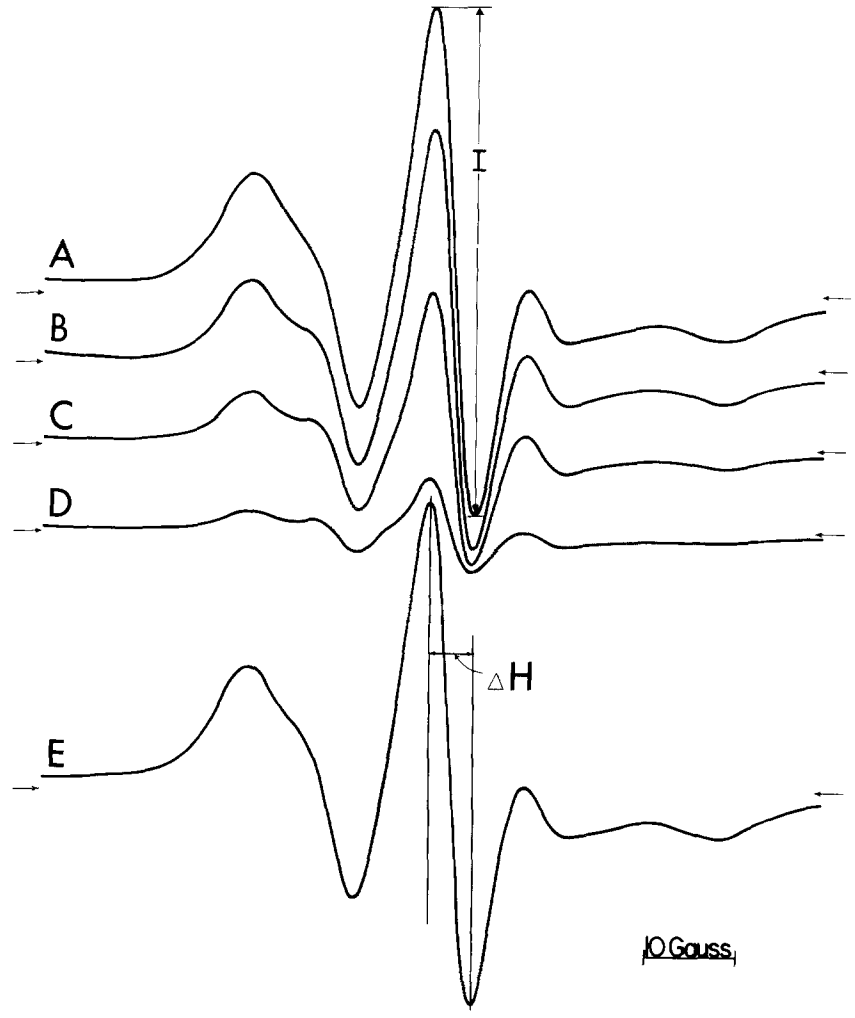


Fig. 3. Time dependence of the ESR spectra of I(12,3)-labeled platelets at 37 °C. The spectra were recorded at various times using a single platelet sample, labeled at a probe/cell ratio of 10 μg I(12,3)/1.5×10^8 cells. The instrument parameters employed to record the spectra were 4 min scan time, 4×10^3 receiver gain and a 100-G field sweep. The spectra were taken at 9 (*A*), 23 (*B*), 38 (*C*), and 54 (*D*) min after placing the sample into the ESR cavity. The bottom spectrum (*E*) was recorded after measuring (*D*) by adding 1 mM $K_3Fe(CN)_6$ and then waiting 4 min for temperature equilibration. The spectra were not normalized, and the baselines for each spectrum were denoted by arrows to the left and right. The central band amplitude (I) and the peak-to-peak distance of the central band (ΔH) were measured as shown in (*A*) and (*E*), respectively

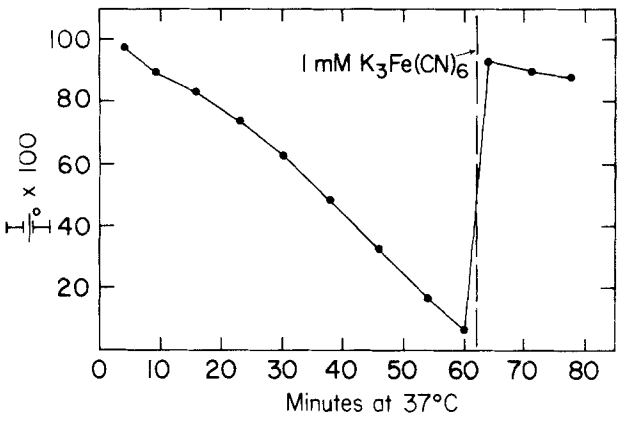


Fig. 4. Time dependence of the amplitude of the central band (I) of I(12,3)-labeled platelets at 37 °C. Platelets were labeled at a probe/cell ratio of 10 μg I(12,3)/1.5×10^8 cells and sequentially recorded spectra were measured as shown in Fig. 3. A plot of I *vs.* time was extrapolated to obtain the initial amplitude at time zero (I_0). $I/I_0 \times 100$ is plotted as the function of the time at which the spectra were recorded. After the 60-min spectrum was measured, the platelet sample was treated with 1 mM $K_3Fe(CN)_6$ and additional spectra were recorded

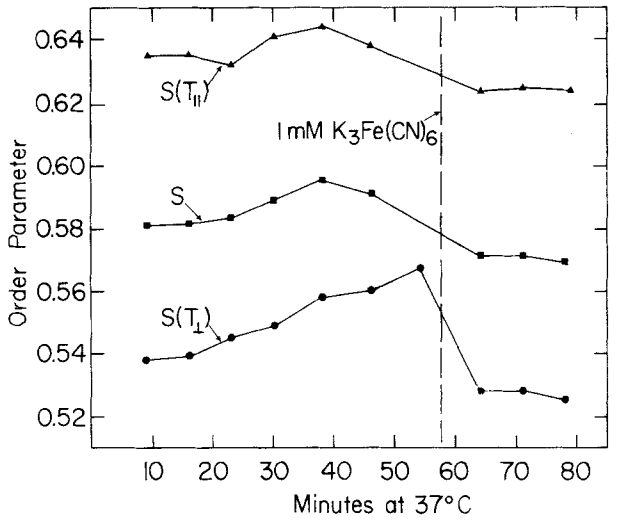


Fig. 5. Time dependence of the order parameters measured from I(12,3)-labeled platelets at 37 °C. The order parameters S [Eq. (3)], $S(T_{\parallel})$ [Eq. (1)], and $S(T_{\perp})$ [Eq. (2)], are plotted as a function of the time of incubation of the labeled cells at 37 °C. The order parameters were calculated by successively recording spectra from the same platelet sample (10 μg I(12,3)/1.5×10^8 cells) as described in the text (*see* Fig. 1 and Materials and Methods). 1 mM $K_3Fe(CN)_6$ was added to the labeled platelets prior to recording the spectrum at 64 min

trum; increasing the distance of the oxazolidine ring from the carboxyl terminus not only decreases the outer hyperfine splitting but also increases the inner splitting. The I(*m*, *n*) labels probably intercalalate into the platelet membrane so that the carboxyl group is relatively anchored to the polar surface, while the more mobile methyl terminus lies within the interior of the membrane. The platelet membrane flexibility gradient, which indicates that the fluidity increases towards the center of the bilayer, is similar to that observed in other I(*m*, *n*)-labeled model and biological membranes (Seelig, 1970; Hubbell & McConnell, 1971; Keith, Sharnoff & Cohn, 1973).

The Effects of Time and Probe Concentration on the Spectra of I(m, n)-Labeled Platelets

Time-dependent decreases in signal intensity were observed in the ESR spectra of human platelets labeled with either the I(12,3), I(5,10) or I(1,14) spin probes. The effects of time on the spectra of I(12,3)-labeled platelets may be seen in Fig. 3; these results were obtained by recording successive spectra from the same sample within 60 min at 37 °C. The signal intensity was greatest for the initially-recorded spectrum (Fig. 3*A*) and appeared to uniformly decrease with time. After recording Fig. 3*D*, 1 mM $K_3Fe(CN)_6$ was added to the I(12,3)-labeled platelet sample and the spectrum was measured following a 4-min wait for temperature equilibration (*see* Fig. 3*E*). The $K_3Fe(CN)_6$ addition almost completely regenerated the ESR signal, suggesting that the signal loss was due to a cell-mediated reduction of the paramagnetic nitroxide label.

It is necessary to consider in more detail the effects of time and $K_3Fe(CN)_6$ on various spectral parameters of I(12,3)-labeled human platelets. Figure 4 is a plot of $I/I_0 \times 100$ *vs.* t(min), where I is the amplitude of the central band measured as shown in Fig. 3*A* and I_0 is the initial I value obtained by extrapolating a plot of I *vs.* t(min) to zero time. The I/I_0 ratio decreased linearly with time at 37 °C and, under the conditions employed for the representative experiment in Fig. 4, I decreased to $I_0/2$ in 37 min. Platelets exhibited this I(12,3) probe-reducing property at 37 °C whether freshly-prepared or incubated at room temperature for as long as 48 hr after blood collection. Although the ESR signal of I(12,3)-labeled platelets declined as a linear function of time for all conditions tested, the signal reduction proceeded less rapidly if: (i) the probe/cell ratio was greater than that used in Fig. 4; or (ii) spin-label studies were performed at temperatures below 30 °C. Control experiments with freshly-prepared I(12,3)-labeled human erythrocyte ghosts indicated no signal reduction at 37 °C.

As indicated earlier, addition of 1 mM $K_3Fe(CN)_6$

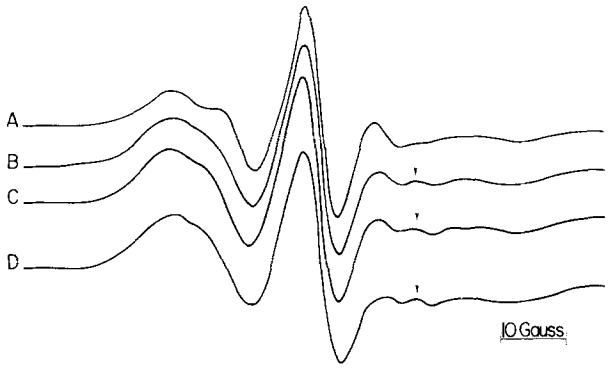


Fig. 6. ESR spectra of platelets labeled with different I(12,3) probe concentrations at 37 °C. The spectra were recorded as described for the spectrum shown in Fig. 1. The probe/cell ratios for (*A*), (*B*), (*C*) and (*D*) were 4, 12, 21, and 36 μg I(12,3)/1.5 × 10^8 cells. The spectra were normalized so that the *I* values were equal. The high field "liquid-line" components arising from probe molecules in solution are indicated by arrows in spectra (*B*)–(*D*). For the platelet concentrations employed here (1.5–2 × 10^6 cells/μl), the immobilized spectrum of the membrane-incorporated I(12,3) probe obscured the low and mid-field contributions of the "liquid-lines"

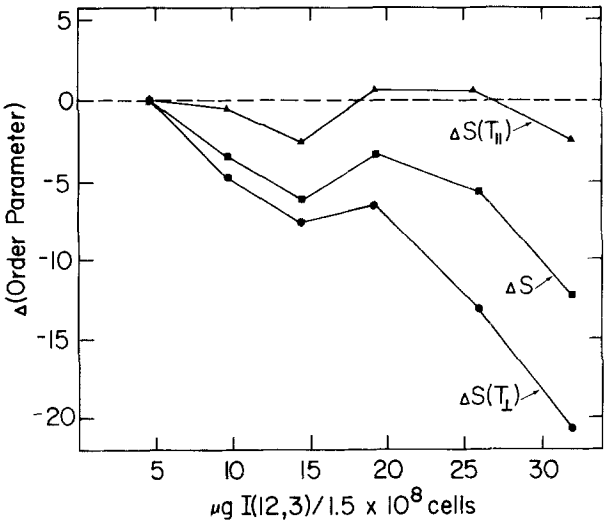


Fig. 7. Probe concentration effects on the order parameters of I(12,3)-labeled platelets at 37 °C. $\Delta S(T_\parallel)$, $\Delta S(T_\perp)$ and ΔS, the percent changes in the order parameters from baseline values measured at 4 μg I(12,3)/1.5 × 10^8 cells, were plotted as a function of the probe/cell ratio. The baseline values of $S(T_\parallel)$, $S(T_\perp)$ and S were 0.647, 0.554, and 0.601, respectively. The order parameters were measured from the spectra of the I(12,3)-labeled platelets as shown in Fig. 1 and described in the text

to I(12,3)-labeled human platelets that had been previously incubated for 60 min at 37 °C quantitatively regenerated the ESR signal. Figure 4 shows that ferricyanide immediately increased the I/I_o ratio from 7 to 94%; moreover, the subsequent signal reduction of I(12,3)-labeled platelets was much less rapid in the presence of ferricyanide. Kaplan, Canonico and Caspary (1973) earlier reported that the time-dependent signal decay of I(*m*, *n*)-labeled mouse L cells could similarly be reversed with ferricyanide addition. Since mouse L cells are relatively impermeable to ferricyanide, Kaplan *et al.* suggested that the spin-labeled molecules primarily reside in the surface membranes. Our own spin-label studies also indicate that a large fraction of the I(12,3) probe in platelets is readily accessible to oxidation by $K_3Fe(CN)_6$. If ferricyanide is excluded from intact human platelets as has been noted for other cells (Mishra & Passow, 1969; Kaplan *et al.*, 1973), then it is likely that the I(12,3) label principally samples the platelet plasma membrane. Additional evidence that suggests that the I(12,3) probe is restricted to the platelet surface membrane is presented in the Discussion.

It is of particular importance to determine whether the time-dependent spectral alterations of I(12,3)-labeled platelets affect either the approximate or polarity-corrected order parameters. Figure 5 indicates that $S(T_\parallel)$ is relatively independent of the ESR signal decrease which occurs in 60 min for platelets labeled with 10 μg I(12,3)/mg protein. However, $S(T_\perp)$, and to a lesser degree S, increased with time; for example, $S(T_\perp)$ and S increased by 3.7 and 2.6% in 40 min. The ESR spectra of I(12,3)-labeled platelets (Fig. 3) exhibit the following alterations with time: (i) $2T_\perp$ decreased while $2T_\parallel$ remained constant; (ii) the high field peak height of the inner hyperfine doublet increased with respect to the spectral baseline; (iii) ΔH, the peak to peak distance of the central band (Sauerheber *et al.*, 1977; Gordon *et al.*, 1978), decreased; (iv) the high field baseline became less depressed while the low field baseline became less elevated; and (v) *I* decreased, as indicated earlier. Qualitatively similar time-dependent spectral changes were obtained for human platelets initially labeled with 4, 20, and 40 μg I(12,3)/1.5 × 10^8 cells.

The most likely interpretation of the order parameter changes in Fig. 5 is that the reduction of I(12,3) probe decreases not only the ESR signal but also the magnitude of probe-probe interactions. We earlier determined that increasing the I(12,3) probe concentration in purified liver or heart plasma membranes induces spectral alterations which are exactly opposite to the changes seen with time in Fig. 5 for labeled platelets; in particular, S and $S(T_\perp)$ decreased while $S(T_\parallel)$ remained constant at appropriately high probe/lipid ratios (Sauerheber *et al.*, 1977; Gordon *et al.*, 1978). The "apparent" increase in the liver and heart membrane fluidity with probe concentration was

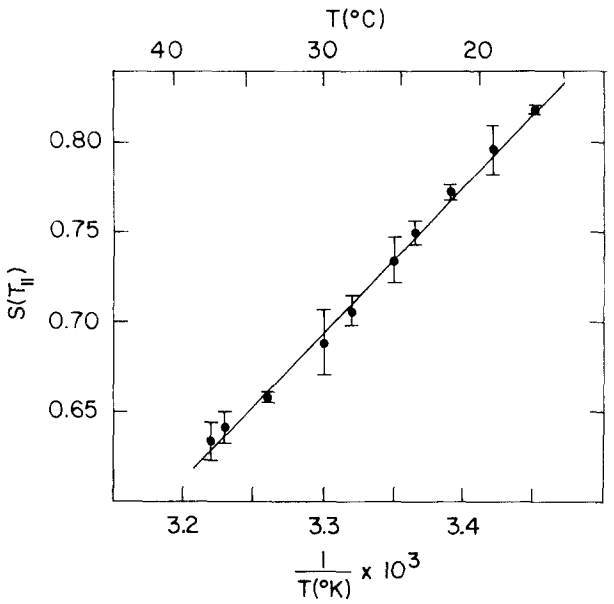


Fig. 8. Temperature dependence of the fluidity of I(12,3)-labeled platelets. The approximate order parameter $S(T_{\|})$ is plotted *vs.* $[1/T(°K)] \times 10^3$ for the range 17 to 37 °C. $S(T_{\|})$ [Eq. (1)] was calculated from the spectra of I(12,3)-labeled platelets (6 μg probe/1.5×10^8 cells) as described in the text. Spectra were recorded, after 4-min waits for temperature equilibration of the sample. Each determination in this figure represents the mean value (±1 SD) obtained from at least three separate platelet preparations; the $S(T_{\|})$ value at 37 °C is the mean determined from 12 separate preparations. A linear regression line having a high coefficient of determination ($r^2 = 0.99$) is drawn through the data

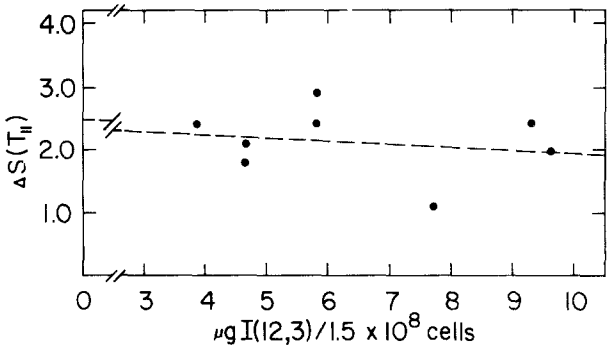


Fig. 9. Effect of calcium on the platelet $S(T_{\|})$ at various probe/cell ratios. $\Delta S(T_{\|})$, the percent change in $S(T_{\|})$ induced by the addition of 3.6 mM $CaCl_2$ to I(12,3)-labeled platelets at 37 °C, is plotted against the probe/cell ratio for the range 3.8 to 9.6 μg I(12,3)/1.5×10^8 cells. $S(T_{\|})$ was calculated from the spectra and Eq. (1) as indicated in the text, and $\Delta S(T_{\|}) = [(S(T_{\|})_2 - S(T_{\|})_1)/(S(T_{\|})_2 + S(T_{\|})_1)] \times 200$. Here, $S(T_{\|})_2$ and $S(T_{\|})_1$ are the order parameters determined before and after Ca^{2+} addition. The mean $S(T_{\|})$ value for the data in this figure was 2.1 ± 0.5%. The linear regression line drawn through the points exhibited a low coefficient of determination ($r^2 = 0.05$) and a negligible slope of −0.05. The extrapolated y intercept at zero probe concentration was +2.3%

attributed to enhanced nitroxide radical interactions rather than to perturbations in the membrane structure. If probe-probe interactions are also initially present in platelets labeled with 10 μg I(12,3)/1.5×10^8 cells at 37 °C, then recording the spectra with time should permit the examination of a "reverse" probe titration in which the concentration of label having unpaired electrons decreases with time.

The hypothesis that the time-dependent order parameter alterations in Fig. 5 are due to changes in radical interactions was tested as follows: 1 mM $K_3Fe(CN)_6$ was added to I(12,3)-labeled platelets after the cells had incubated for 60 min at 37 °C. The ferricyanide treatment completely restored the ESR signal, immediately reduced $S(T_{\perp})$ and S to slightly below basal values, but did not affect $S(T_{\|})$. On the other hand, control ESR spectra of I(12,3)-labeled platelets [10 μg I(12,3)/1.5×10^8 cells], recorded from the cells incubated at 37 °C for only 4 min, were not altered by the addition of either 1 or 2 mM $K_3Fe(CN)_6$. Although the total concentration of reduced and unreduced probe in the platelet membrane is apparently independent of time, the ratio of reduced to unreduced probe in Fig. 5 increased with time and decreased upon ferricyanide addition. It is unlikely, therefore, that the changes observed in $2T_{\perp}$ with time are simply due to probe-mediated perturbations in the membrane structure. The invariance of $S(T_{\|})$ (or $2T_{\|}$) in Fig. 5 also argues against an alteration in the membrane structure; any fluidization which permits more flexibility in the membrane-incorporated probe requires that $T_{\|}$ commensurately decrease with increases in $2T_{\perp}$.

The effects of a range of I(12,3) probe concentrations on the spectra and order parameters of labeled platelets were also examined. ESR spectra of platelet samples containing widely different I(12,3) probe concentrations at 37 °C are shown in Fig. 6. Each spectrum was recorded from a separately labeled platelet sample after a 4-min wait at 37 °C; this procedure minimized any interference due to the time-dependent signal losses described above. Increasing the probe/cell ratio decreased the high-field peak of the inner hyperfine doublet, displaced downward the high-field baseline and upward the low-field baseline, increased $2T_{\perp}$ and ΔH [the peak to peak distance of the central band (Sauerheber *et al.*, 1977; Gordon *et al.*, 1978)] and left $2T_{\|}$ unchanged. In addition, a small "liquid-line" appeared at the high probe/lipid ratios (*see* arrows in Fig. 6) due to a portion of the probe tumbling rapidly in an aqueous environment. Order parameters were also determined from spectra obtained from platelets labeled with different I(12,3) probe concentrations. Figure 7 is a plot of Δ(order parameter) *vs.* probe/cell ratio, where Δ(order parameter) is the percent change in the platelet order para-

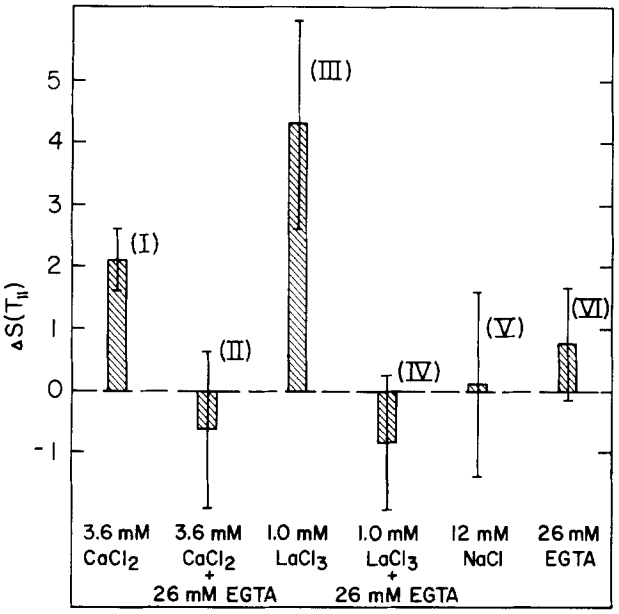


Fig. 10. Effects of various agents on the fluidity of I(12,3)-labeled platelets at 37 °C. The relative effects on the platelet $S(T_{\parallel})$ by additions of 3.6 mM $CaCl_2$ (data in Fig. 9) (*I*), 1 mM $LaCl_3$ (data in Fig. 11) (*III*), 12 mM NaCl (*V*), 26 mM EGTA (*VI*) and premixed solutions containing $CaCl_2$ and EGTA (*II*) or $LaCl_3$ and EGTA (*IV*) are shown. The number of separate determinations for *I*, *II*, *III*, *IV*, *V* and *VI* were 8, 6, 9, 6, 5 and 4; the probe/cell ratios for *II*, *IV*, *V* and *VI* were 6, 10, 6, and 6 μg I(12,3)/1.5×10^8 cells. $\Delta S(T_{\parallel})$ is the percent difference in $S(T_{\parallel})$ induced by the perturbing agent and is calculated as described in Fig. 9. The mean values for the effects due to each of the added agents were statistically compared with the two-sample *t* test of significance for unequal sample sizes (Kilpatrick, 1977). *P* values, tabulated for the following sample pairs, were negligible, suggesting that the sample means are significantly different: *III* and *IV*, $P<0.001$; *III* and *I*, $P<0.005$; *II* and *I*, $P<0.001$; *I* and *V*, $P<0.02$; and *I* and *VI*, $P<0.01$. *P* values tabulated for the following pairs of sample means did not suggest significant differences: *IV* and *VI*, $P>0.02$; *II* and *VI*, $P>0.05$; and *II* and *IV*, $P>0.2$

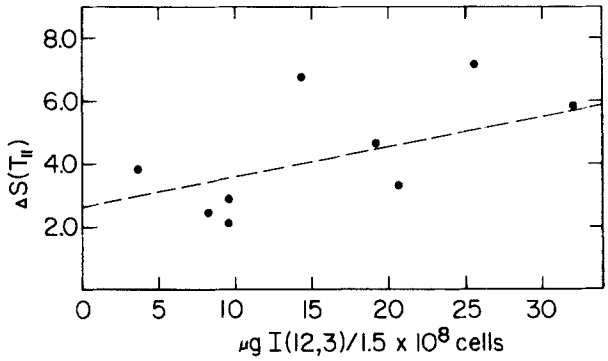


Fig. 11. Effect of $LaCl_3$ on the platelet $S(T_{\parallel})$ for different I(12,3) probe/cell ratios. $\Delta S(T_{\parallel})$, the percent increase in $S(T_{\parallel})$ induced by addition of 1 mM $LaCl_3$ to I(12,3)-labeled platelets at 37 °C, is plotted *vs.* the probe/cell ratio for the range 3.8 to 35 μg I(12,3)/1.5×10^8 cells. The mean value was $+4.3 \pm 1.7\%$. $S(T_{\parallel})$ was calculated from the spectra as indicated in the text. A linear regression analysis of the data in this figure yielded a line with a small positive slope and a low coefficient of determination of $r^2=0.4$. The *y* intercept was +2.6%

meter from baseline values measured at 4 μg I(12,3)/1.5×10^8 cells. Here, $\Delta S(T_{\perp})$ and ΔS decreased with increasing probe concentration, while $\Delta S(T_{\parallel})$ remained unchanged throughout the probe range tested. Both the spectral and order parameter changes induced by increasing probe concentration in intact platelets are in good agreement with similar studies performed in isolated liver and heart plasma membranes at high probe/lipid ratios (Sauerheber *et al.*, 1977; Gordon *et al.*, 1978). Moreover, the spectral broadening and splitting changes which occur with increasing probe concentration in I(12,3)-labeled platelets are exactly opposite to the time-dependent spectral alterations seen in Figs. 3 and 5. The most likely explanation of the spectral and order parameter effects in Figs. 6 and 7 is that they are the result of enhanced nitroxide radical interactions.

The major conclusion from these experiments is that $S(T_{\parallel})$ is a suitable parameter to monitor the fluidity of I(12,3)-labeled intact platelets. $S(T_{\parallel})$ remained constant despite large changes in nitroxide radical interactions. Conversely, *S* and $S(T_{\perp})$ were judged unsatisfactory measures of membrane fluidity, since these parameters exhibited time-dependent changes which were associated with alterations in probe-probe interactions. We previously determined that the presence of nitroxide radical interactions might generally interfere with the measurement of intrinsic membrane properties (Sauerheber *et al.*, 1977). Here, intrinsic membrane properties are defined as those which are measured when probe-probe interactions are negligible and do not refer to membrane behavior in the absence of a perturbing spin label. Ideally, intrinsic properties may be assessed in those cases where "magnetically-dilute" concentrations are established from probe titration experiments. Unfortunately, intrinsic *S* and $S(T_{\perp})$ values could not be detected with I(12,3)-labeled whole platelets; nitroxide radical interactions are probably present in the lowest probe/cell ratio which could be tested due to signal/noise limitations. Nevertheless, $S(T_{\parallel})$ undoubtedly reflects an "intrinsic", albeit polarity-uncorrected, membrane fluidity since this parameter was insensitive to both increasing time and probe concentration.

Temperature Effects on Platelet Membrane Fluidity

The temperature dependence of the fluidity of I(12,3)-labeled platelets was examined for the range 17 to 37 °C; a plot of $S(T_{\parallel})$ *vs.* $1/T(°K)$ is shown in Fig. 8. Electron spin resonance spectra of the labeled samples

were recorded by progressing from low to high temperatures. $S(T_{\parallel})$ measurements for each temperature in Fig. 8 below 30 °C could be obtained from a single I(12,3)-labeled platelet sample, due to the limited ESR signal decay. However, the more rapid rates of I(12,3) probe reduction present at higher temperatures required the use of several platelet samples in order to obtain comparably accurate $S(T_{\parallel})$ values. Each $S(T_{\parallel})$ determination in Fig. 8 represents the mean value obtained from at least three separate platelet preparations. The Arrhenius-type plot of $S(T_{\parallel})$ indicates that the fluidity of platelet membranes decreased linearly with increasing $1/T(°K)$ with a high coefficient of determination. The $S(T_{\parallel})$ value of I(12,3)-labeled platelets, which decreases with increasing temperature, thus appears to be a sensitive monitor of temperature-dependent changes in the lipid fluidity.

Calcium Effects on the Platelet Membrane Fluidity

The influence of calcium on the platelet membrane fluidity was investigated at 37 °C with probe/cell ratios ranging from 4 to 10 μg I(12,3)/1.5×10^8 platelets. Addition of 3.6 mM $CaCl_2$ decreased the platelet membrane fluidity, as indicated by positive increases in $\Delta S(T_{\parallel})$ in Fig. 9 for each probe concentration tested; here $\Delta S(T_{\parallel})$ was calculated as the percent difference between values measured with and without Ca^{2+}. The plot of $\Delta S(T_{\parallel})$ *vs.* probe/cell ratio in Fig. 9 shows that the Ca^{2+}-dependent rigidization of the platelet membrane was independent of the probe/cell ratio; probe concentrations were selected from a range in which radical interactions substantially increased (*see* Fig. 7). A linear regression line drawn through the data in Fig. 9 was characterized by not only a small coefficient of determination but also a negligible slope. Furthermore, the mean increase in $S(T_{\parallel})$ [i.e., $\Delta S(T_{\parallel}) = +2.1 \pm 0.5\%$] induced by Ca^{2+} for the eight platelet samples used in Fig. 9 agreed well with the extrapolated y intercept (i.e., $\Delta S(T_{\parallel}) = +2.3\%$]. The y intercept reflects the magnitude of the Ca^{2+} effect at zero probe concentration. It should be noted that the decrease in membrane fluidity at 37 °C induced by 3.6 mM $CaCl_2$ is similar in magnitude to the effect of lowering the temperature of the labeled cells from 37 to 35 °C.

The nature of the $CaCl_2$-induced rigidization of the I(12,3)-labeled platelets at 37 °C was characterized in additional experiments. To test the possibility that the calcium effect was simply due to an increase in the ionic strength of the medium, we examined the alterations induced by NaCl on the order parameter $S(T_{\parallel})$ of the spin-labeled platelets. Addition of 12 mM NaCl did not significantly affect $S(T_{\parallel})$ (Fig. 10); moreover, the mean $\Delta S(T_{\parallel})$ value obtained after the addition of 3.6 mM $CaCl_2$ was significantly greater than the corresponding value found after addition of 12 mM NaCl. Similar studies performed on I(12,3)-labeled human erythrocytes indicated that NaCl (50–200 mM) also did not affect the outer hyperfine splitting $2T_{\parallel}$ (Rigaud, Gary-Bobo & Taupin, 1974). These results suggest that the Ca^{2+}-mediated decrease in platelet fluidity is probably due to the binding of Ca^{2+} to specific membrane sites.

The $CaCl_2$ effects on the platelet membrane fluidity at 37 °C were also found to be reversed by the use of EGTA, a calcium-chelating agent. 26 mM EGTA when added to a I(12,3)-labeled platelet sample which initially contained 3.6 mM $CaCl_2$, reduced the fluidity [i.e., the mean $\Delta S(T_{\parallel})$ was equal to $-2.2 \pm 1.0\%$ from 3 experiments]. Alternatively, a similar addition of EGTA to I(12,3)-labeled platelet samples without Ca^{2+} did not change the membrane fluidity (Fig. 10). In further studies, no alterations in the fluidity were detected when an aliquot of a premixed solution of $CaCl_2$ and EGTA was added to the labeled platelets to yield final Ca^{2+} and EGTA concentrations of 3.6 and 26 mM; here, the mean $\Delta S(T_{\parallel})$ determined after addition of 3.6 mM $CaCl_2$ was significantly greater than that induced by the premixed $CaCl_2$-EGTA solution (Fig. 10). Thus, the effects of Ca^{2+} on the membrane fluidity could, under appropriate conditions, be either reversed or prevented with EGTA. Unfortunately, the marked ESR signal decay at 37 °C prohibited the sequential measurement of a control spectrum, a spectrum after $CaCl_2$ addition, and then a spectrum after EGTA addition using the same I(12,3)-labeled platelet sample.

$LaCl_3$ Effects on Platelet Fluidity and Clumping

Experiments were also performed to assess the effects of La^{3+} on the platelet membrane fluidity. The addition of 1 mM $LaCl_3$ decreased the fluidity of platelets labeled with probe concentrations ranging from 3.8 to 32 μg I(12,3)/1.5×10^8 cells, as indicated by the positive $\Delta S(T_{\parallel})$ values in Fig. 11. Similar to the results obtained with $CaCl_2$, Fig. 11 shows that the effect of 1 mM La^{3+} on the platelet fluidity was essentially independent of the probe/cell ratio over a wide range. A linear regression analysis of the data in the $\Delta S(T_{\parallel})$ *vs.* probe/cell ratio plot in Fig. 11 yielded a low coefficient of determination and a line with a slight positive slope. It should be noted that the mean $\Delta S(T_{\parallel})$ of the nine determinations in Fig. 11 agreed with the extrapolated y intercept to within experimental error (i.e., $+4.3 \pm 1.7\%$ *vs.* $+2.6\%$). The increase in $S(T_{\parallel})$

induced by 1 mM La^{3+} was significantly greater than the effects caused by either 3.6 mM $CaCl_2$ or 12 mM NaCl. A decrease in the platelet temperature from 37 to 33 °C would be necessary to increase $S(T_{\parallel})$ by the same amount seen with addition of 1 mM $LaCl_3$ at 37 °C. A reasonable interpretation of these results is that La^{3+} rigidizes the platelet membrane by binding to specific membrane sites.

Studies were also conducted to determine whether the La^{3+} perturbations on the platelet fluidity were reversible. 26 mM EGTA, when added to a I(12,3)-labeled platelet sample containing 1 mM $LaCl_3$, decreased the fluidity at 37 °C [i.e., the mean $\Delta S(T_{\parallel}) = -4.2 \pm 2\%$, obtained from 3 experiments]. As indicated earlier, the addition of 26 mM EGTA to a I(12,3)-labeled platelet sample without $LaCl_3$ did not significantly affect the membrane fluidity. Moreover, no change in the fluidity occurred when an aliquot of a premixed solution of $LaCl_3$ and EGTA was added to labeled platelets to yield final La^{3+} and EGTA concentrations of 1 and 26 mM; the mean $\Delta S(T_{\parallel})$ induced by 1 mM La^{3+} was significantly larger than that mediated by the premixed La^{3+}-EGTA solution (Fig. 10). These data suggest that La^{3+} decreases the platelet fluidity by reversibly binding to membrane sites.

The conditions under which La^{3+} induced platelet clumping were examined by viewing platelet samples (approximately 7×10^5 cells/μl) in siliconized 50 μl pipettes against a dark background. $LaCl_3$ additions (0.2 to 1 mM) caused rapid clumping of either I(12,3)-labeled or unlabeled platelets if no adenosine was present in the Tris buffer. Subsequent treatment with 26 mM EGTA reversed the La^{3+}-induced platelet clumping. However, no precipitation was observed for La^{3+} concentrations between 0.2 and 3 mM if the Tris buffer also contained the aggregation-inhibiting agent adenosine (30 mM). All of the membrane ESR studies were performed with adenosine in the buffer, inasmuch as platelet clumping interfered with the spectral measurements.

Discussion

The Cellular Location of the I(m, n) Probe in the Whole Human Platelets

Spin-labeled derivatives of stearic acid, I(*m*, *n*), have been widely employed to investigate the lipid structural properties of either intact cells (Kaplan *et al.*, 1973; Gaffney, 1975) or purified membrane preparations (Breton *et al.*, 1977; Sauerheber *et al.*, 1977; Gordon *et al.*, 1978). An important question in I(*m*, *n*)-labeled whole cell studies concerns whether the probe partitions into membranes of intracellular organelles (e.g., endoplasmic reticulum, mitochondria, etc.) as well as surface membranes. If the I(*m*, *n*) label is not exclusively present in the plasma membrane, then the ESR spectra will reflect a fluidity which is averaged over the various membrane environments sampled by the probe. Obviously, knowledge of the relative distribution of the spin probe in the cellular membranes would greatly assist in the interpretation of the spectra of I(*m*, *n*)-labeled cells.

Both direct and indirect evidence indicates that the I(12,3) probe principally resides in the plasma membrane of whole platelets. Gaffney (1975) earlier suggested, in a study of the fluidities of normal and transformed mouse fibroblasts, that a substantial portion of the I(*m*, *n*) label was in the plasma membrane since: (i) measurements were made within several minutes of introduction of label; and (ii) rapid cellular destruction of the label occurred. These arguments could also be invoked to assign a surface membrane location for I(*m*, *n*)-labeled platelets, since our experimental conditions were similar to the labeled fibroblast study. It should be noted that the partitioning of the probe throughout the plasma membrane and surface-connected open canalicular system (SCCS) in whole platelets would be expected to be complete within a few seconds, since previous I(*m*, *n*) studies have demonstrated a rapid translational motion of the probe in both model and biological membranes (i.e., diffusion constant approx. 10^{-8} cm^2/sec) (Lee, 1975; Jain & White, 1977). Contrarily, the platelet internal membranes would be much less likely to be labeled by lateral diffusion of the probe from the plasma membrane; here, the organelle zone in platelets (i.e., consisting of granules, electron-dense bodies, mitochondria, and elements of the dense tubular system) is physically separated from both the surface membrane and the SCCS (White & Gerrard, 1976). Another mechanism by which the subcellular organelles of platelets might be labeled would be for the I(12,3) probe to first "flip-flop" from the outer to the inner half of the plasma membrane, and then diffuse through the cytosol into the organelle zone. The redistribution of label by a "flip-flop" process is not likely in platelets, in view of the erythrocyte studies of Devaux and coworkers which showed that a phospholipid label exhibited negligible transverse diffusion at 25 °C (Rousselet *et al.*, 1976).

Studies have also been performed with various agents in this and other laboratories which suggest that the I(12,3) label resides in the surface membrane of platelets. As discussed earlier, the reversal of the time-dependent signal decay of I(12,3)-labeled intact platelets with ferricyanide (Figs. 3 and 4) indicates that the probe is primarily located in the plasma mem-

Table 1. Order parameter values for biological membranes labeled with magnetically-dilute concentrations of I(12,3) at 30 °C

Membrane system[a]	$S(T_{\parallel})$	S	$S(T_{\perp})$
Rat liver plasma membrane	0.635 ± 0.008	0.615 ± 0.006	0.590 ± 0.005
Rat heart plasma membrane	0.660 ± 0.009	0.625 ± 0.006	0.595 ± 0.006
Human platelets	0.688 ± 0.020	–	–
Human platelet plasma membrane	0.705 ± 0.010	0.655 ± 0.006	0.605 ± 0.010
Human erythrocyte ghosts	0.745 ± 0.006	0.674 ± 0.005	0.618 ± 0.005

[a] All determinations were performed at physiological pH with samples suspended in Tris buffer. Rat liver and heart plasma membranes were prepared and labeled as described previously (Sauerheber *et al.*, 1977; Gordon *et al.*, 1978). Human platelet plasma membranes, purified according to Barber and Jamieson (1970), and human erythrocyte ghosts, prepared as described by Dodge, Mitchell and Hanahan (1963), were labeled with 11 and 3 μg I(12,3)/mg membrane protein, respectively. Human platelets were isolated as described in the text (*see* Materials and Methods) and were labeled with 10 μg I(12,3)/1.5×10^8 cells. S, $S(T_{\parallel})$ and $S(T_{\perp})$ were calculated as indicated in Materials and Methods.

brane. The decrease in the fluidity of I(12,3)-labeled platelets mediated by 1 mM $LaCl_3$ (Figs. 10 and 11) also suggests that the I(12,3) probe is present in the plasma membrane, since La^{3+} generally does not penetrate intact cells (Robblee & Shepro, 1976; Weiss, 1974; Mikkelsen, 1976). Previous electronmicroscopic investigations of platelets treated with $LaCl_3$ indicate that La^{3+} accumulates only at the surface membrane and the SCCS (White & Gerrard, 1976). The fact that lanthanum and ferricyanide, agents normally excluded from the interior of intact cells, dramatically alter the spectra of I(12,3)-labeled whole platelets in very different ways indicates that the probe is restricted to the cell surface. Lastly, incubation of human platelets with a ^{3}H-palmitate-albumin solution was found in autoradiographic studies to result in preferential incorporation of the label into the surface membrane (Hoak *et al.*, 1972). It is possible, then, that the 5-nitroxide stearic acid derivative might similarly partition into only the platelet plasma membrane.

Preliminary spin-label experiments conducted on purified platelet plasma membranes also suggest that the I(12,3) probe is present only in the surface membranes of intact platelets. Platelet plasma membranes were purified following the procedure of Barber and Jamieson (1970) by lysing glycerol-loaded platelets and then centrifuging lysates through 25% wt/vol sucrose. The putative plasma membrane fraction, which collected at the interface of the loading medium and the 25% wt/vol sucrose, exhibited highly-enriched Na^+,K^+-ATPase (approx. 3.1-fold over that in lysate) and acid phosphatase (approx. 3.9-fold) activities. Electronmicroscopic analysis of the plasma membrane fraction indicated the presence of both double and single membrane vesicles with diameters similar to those reported previously (Barber & Jamieson, 1970). The ESR signal of platelet plasma membranes labeled with a low I(12,3) probe concentration did not decay at 37 °C after several hours. Nevertheless, the $S(T_{\parallel})$ values obtained from the I(12,3)-labeled isolated membranes for the range 17 to 37 °C were generally in good agreement with the corresponding order parameters obtained from I(12,3)-labeled intact platelets (*see* Table 1 for order parameters at 30 °C). Moreover, the use of either 3.6 mM $CaCl_2$ or 1 mM $LaCl_3$ decreased the fluidity of I(12,3)-labeled plasma membranes at 37 °C to the same degree seen with the whole cells. These results would be predicted if the probe was localized in the plasma membrane of I(12,3)-labeled intact platelets.

The Fluidity of I(12,3)-Labeled Platelets: Physiological Significance and Comparison with the Fluidities of Other Biological Membranes

The surface membrane plays central roles in regulating a variety of platelet physiological processes, including the adhesion of platelets to nonendothelial surfaces and the aggregation and release reaction of platelets (Cooper, Mason & Brinkhous, 1976; Crawford & Taylor, 1977). Ultrastructural analysis of the platelet surface membrane with electronmicroscopy indicates a plasma membrane with a thickness of 70 to 90 Å and a fluffy coat of 150 to 250 Å extending into the extracellular medium (Mustard & Packham, 1970). The platelet plasma membrane consists of a heterogeneous mixture of proteins, phospholipids and neutral lipids while the surface coat is believed to contain proteins and sulfated acid mucopolysaccharide (Mustard and Packham, 1970; Cooper *et al.*, 1976; Crawford & Taylor, 1977). The spectra of I(*m*, *n*)-labeled platelets, which demonstrate that the spin probes undergo rapid anisotropic motion about their long molecular axes, indicate that the labels distribute into the fluid lipids of the plasma membrane rather than the fluffy surface coat. Furthermore, the platelet

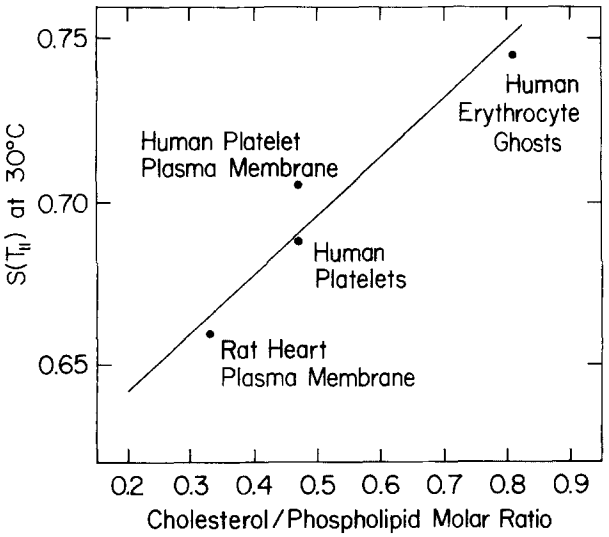


Fig. 12. Plot of $S(T_{\parallel})$ *vs.* the cholesterol/phospholipid molar ratio (*C/Pl*) for various I(12,3)-labeled biological membranes at 30 °C. The *C/Pl* ratio used for erythrocytes was determined by Dodge and Philipps (1967), while that used for the platelet membranes is an average of the values determined by Barber and Jamieson (1970) for the platelet plasma membrane subfractions. The *C/Pl* ratio of rat heart plasma membranes was measured by Feldman and Weinhold (1977). There is, in general, good agreement in the literature for the *C/Pl* ratios of erythrocytes (0.81 to 0.90) and platelet plasma membranes (0.45 to 0.50). However, the data for liver plasma membranes is not included here due to the wide scatter in the reported *C/Pl* ratios (0.26 to 0.70) (Evans, 1970); possibly the divergent *C/Pl* ratios for liver membranes are due to the differences in the purity of the preparations. $S(T_{\parallel})$ was calculated as indicated in Table 1 and the text

plasma membrane exhibits the familiar "fluidity gradient", since the flexibility of the hydrocarbon backbone is greater near the methyl terminus of the label (Figs. 1 and 2). Thus, the internal lipid regions of the platelet membrane bilayer are probably more fluid and disordered than regions nearer the polar membrane surface.

The fluidity of I(12,3)-labeled intact platelets may be compared with the corresponding fluidities of other, similarly-labeled, biological membranes. The intrinsic S, $S(T_{\parallel})$ and $S(T_{\perp})$ values of rat liver and heart plasma membranes, and human platelet and erythrocyte ghost plasma membranes, were obtained by using "magnetically-dilute" I(12,3) probe concentrations at 30 °C (Table 1). Probe titrations were conducted on each plasma membrane following a protocol which insures that nitroxide radical interactions will not affect the observed hyperfine splittings (Sauerheber *et al.*, 1977). In the case of I(12,3)-labeled intact platelets, the presence of radical interactions for all probe/lipid ratios tested prevented the measurement of intrinsic S and $S(T_{\perp})$ values. However, the $S(T_{\parallel})$ of whole platelets was independent of the probe concentration and is included in Table 1. Examination of the respective polarity-corrected order parameters (S) of the plasma membrane systems in Table 1 indicates that the fluidities decrease in the following order: liver membranes > heart membranes > platelet membranes > erythrocyte ghosts. Similar relative fluidities were found if either $S(T_{\parallel})$ or $S(T_{\perp})$ was employed as the index of the flexibility of the probe. Although $S(T_{\parallel})$ and $S(T_{\perp})$ do not correct for polarity differences between the membrane and the reference crystal, these approx. order parameters appear to be useful measures of the lipid fluidity for the above biological membranes. Table 1 also shows that the $S(T_{\parallel})$ values of intact human platelets and purified platelet plasma membranes agree to within experimental error. If it be assumed that the polarity corrections to the $S(T_{\parallel})$ values of intact platelets and platelet plasma membranes are similar, then Table 1 indicates that the flexibility of the probe in the whole cells is the same as that in the isolated membranes. Moreover, the fluidity of I(12,3)-labeled intact platelets at 30 °C is probably less than that of rat liver and heart plasma membranes, but greater than that of erythrocyte ghosts.

It is worthwhile to consider what the structural basis might be for the membrane fluidity differences observed in Table 1. Investigators have earlier demonstrated that membrane-incorporated I(*m*, *n*) labels are sensitive to such parameters as: (i) the Ca^{2+} and Mg^{2+} content (Butler *et al.*, 1970; Ehrström *et al.*, 1973; Sauerheber & Gordon, 1975; Marsh *et al.*, 1976; Uyesaka *et al.*, 1976; Breton *et al.*, 1977; Weller & Haug, 1977; Gordon *et al.*, 1978); (ii) the protein to phospholipid ratio (Jost *et al.*, 1970); (iii) the unsaturated to saturated fatty acid ratio of constituent lipids (Gaffney, 1975); (iv) the membrane temperature; and (v) the cholesterol/phospholipid (*C/Pl*) ratio (Jain, 1975; Bales & Leon, 1978). The heterogeneity of the membrane structure listed in Table 1 largely prevents a rigorous interpretation of how each of the above factors contributes to the observed order parameters. One particularly important problem concerns the distribution of the I(12,3) probe in the membrane. I(*m*, *n*) labels have been found in several binary phase, model lipid systems to partition preferentially into the more fluid phase (Oldfield & Chapman, 1972; Oldfield, Keough & Chapman, 1972; Butler *et al.*, 1974). If the I(12,3) probe is similarly excluded from any solid lipid domains that exist in

the membranes of Table 1, the calculated order parameter might indicate an unusually fluid environment which is not representative of the entire membrane.

Nevertheless, it may prove useful to assess the possible role which the cholesterol content plays in regulating the lipid fluidity. $S(T_{\|})$ was accordingly plotted against the *C/Pl* ratio for the I(12,3)-labeled membranes of Table 1 (Fig. 12); the *C/Pl* ratios were obtained from literature values as described in the figure legend. Enhanced cholesterol contents appear to be associated with decreases in the membrane fluidity, since Fig. 12 shows that $S(T_{\|})$ increases with the *C/Pl* ratio. Bales and Leon (1978) observed a similar dependence of *S* on the *C/Pl* ratio in a review of several I(12,3)-labeled whole cell studies, although "magneticallydilute" probe concentrations were not established here with probe titration experiments. In a recent study of platelets labeled with the fluorescence probe, 1,6-diphenyl-1,3,5-hexatriene (DPH), Shattil and Cooper (1976) provided additional evidence that the membrane fluidity might be affected by the *C/Pl* ratio. Incubation of platelets with various cholesterol-lecithin sonicated dispersions not only altered the plasma membrane *C/Pl* ratio but also modulated the lipid fluidity. Although these data suggest that the *C/Pl* ratio may be involved in the fluidity differences in Table 1, additional experiments are obviously required in order to elucidate any relationship.

Several recent studies have suggested that the relative fluidity of the platelet plasma membrane may have functional significance. For example, fluorescence polarization experiments indicated that changes in the platelet membrane fluidity were associated with alterations in platelet aggregability (Shattil *et al.*, 1975; Shattil & Cooper, 1976). Cooper and coworkers (Shattil *et al.*, 1975; Shattil & Cooper, 1976) observed that platelets, whose plasma membrane *C/Pl* ratio was increased by treatment with cholesterol-rich liposomes, not only exhibited an increased lipid microviscosity but also were more sensitive than normal to the aggregating agents ADP and epinephrine. Alternatively, incubation of platelets with cholesterol-poor liposomes decreased the plasma membrane *C/Pl* ratio, lowered the responsiveness of the cells to the above aggregating agents, and increased the membrane fluidity. It is also of interest that Colman *et al.* (1977) found that the ability of eleven amantadine derivatives to induce phase separations in dipalmitoyl lecithin model membranes correlated with their relative potencies in inhibiting platelet aggregation. Moreover, Poste and Allison (1973) recently suggested that the lipid fluidity may be important in regulating the fusion of intracellular granules with the surface membranes of cells which undergo stimulus-secretion coupling. Since the platelet-release reaction is an exocytotic process in which granules containing agents such as serotonin, ADP, etc., fuse with the cell surface, a normal platelet response to release-inducing agents may require an appropriately fluid plasma membrane.

The Effects of Temperature on Platelet Membrane Fluidity and Function

The studies presented here indicate that the fluidity of I(12,3)-labeled platelets decreased as the temperature is lowered from 37 to 17 °C, since $S(T_{\|})$ increased linearly with increasing $1/T(°K)$ for this temperature range. It should be noted that the effects of temperature on the lipid fluidity have been examined for a wide variety of I(*m*, *n*)-labeled biological membranes. In many instances, characterisitc "breaks" or "discontinuities" have been identified in Arrhenius plots of the order parameters and have been attributed to either a lipid phase transition or separation (Grisham & Barnett, 1973; Houslay *et al.*, 1976*a*; Viret & Letterrier, 1976; Gordon *et al.*, 1978). The absence of any "break" in Fig. 8 might be interpreted to mean that a phase separation does not occur in labeled intact platelets. In a fluorescence study of platelets labeled with DPH, Shattil and Cooper observed that the logarithm of the platelet microviscosity increased linearly with increases in the reciprocal of the temperature (Shattil & Cooper, 1976); these results also might suggest that the fluorescent probe does not "sense" any platelet membrane phase separation or transition.

Nevertheless, we wish to emphasize that neither our spin-label investigation nor the above fluorescence studies prove that a lipid phase separation does not occur in platelet plasma membranes for this temperature range. A major difficulty in the interpretation of Arrhenius-type plots of motional parameters of membrane-incorporated labels concerns the distribution of the probe. Both I(*m*, *n*) labels (Oldfield *et al.*, 1971; Oldfield & Chapman, 1972; Butler *et al.*, 1974) and various fluorescence probes (Bashford, Morgan & Radda, 1976) have been found in binary phase, model lipid systems to preferentially distribute into the more fluid phase. If either I(12,3) or DPH were to be restricted to the more liquid lipid domains in platelets, phase changes occurring in the more solid lipid domains would not necessarily be detected in Arrhenius-type plots of motional parameters of the probes.

The temperature effects on the platelet membrane fluidity are of particular interest in view of the fact that chilling platelets profoundly influences various cellular functions. The lipid fluidity, in general, has been proposed to play important roles in modulating

membrane-associated processes (Lee, 1975; Jain & White, 1977; Gordon *et al.*, 1978). For example, the thermotropic lipid phase separation observed in I(12,3)-labeled rat liver plasma membrane (between 19 and 28 °C) might, in part, be responsible for the effects that temperature is known to exert on insulin degradation and glucose transport rates in liver cells (Gordon *et al.*, 1978), and the activation of such plasma membrane enzymes as Na^+,K^+, ATPase and glucagon-stimulated adenylyl cyclase (Houslay *et al.*, 1976*a*, *b*; Gordon *et al.*, 1978). The lipid phase separation observed in I(12,3)-labeled rat heart plasma membranes (between 21 and 32 °C) might similarly affect various ion transport processes mediated by the sarcolemma (Gordon *et al.*, 1978). In the case of platelets, incubation at cold temperatures induces a shape change closely resembling that caused by ADP at 37 °C (White & Krivit, 1967); moreover, the use of low temperatures may induce platelets to either spontaneously aggregate or to become hyperaggregable in response to ADP additions (Breddin *et al.*, 1977; White, 1977). Although it has been proposed that these temperature effects may be simply due to conformational changes in platelet membrane proteins (Katlove & Alexander, 1971), our results also suggest the possibility that temperature-mediated decreases in the lipid fluidity are involved.

The Interaction of Ca^{2+} and La^{3+} with I(12,3)-Labeled Human Platelets: Effects on Membrane Fluidity

Calcium decreases the fluidities of a wide variety of I(12,3)-labeled biological membranes, including rat liver and heart plasma membranes (Sauerheber & Gordon, 1975; Sauerheber *et al.*, 1977; Gordon *et al.*, 1978), rat brain myelin, synaptosomal membranes and synaptic vesicles (Viret & Leterrier, 1976; Breton *et al.*, 1977), chromaffin granules from bovine adrenal medulla (Marsh *et al.*, 1976), *Bacillus subtilis* cytoplasmic membranes (Ehrström *et al.*, 1973), and membranes of the mycoplasma *Thermoplasma acidophila* (Weller & Haug, 1977). The membrane fluidity in each case was followed by calculating one or both of the hyperfine splittings of the labeled membranes. In the present study, addition of 3.6 mM Ca^{2+} decreased the membrane fluidity of I(12,3)-labeled human platelets, as indicated by positive changes in the order parameter $S(T_\parallel)$. The polarity-corrected order parameter (S) could not be employed as an index of platelet membrane fluidity, due to the dependence of the inner hyperfine splitting, $2T_\perp$, on time and probe concentration at 37 °C. It should be pointed out that $S(T_\parallel)$ is a function of both the polarity and fluidity of the membrane (Gordon & Sauerheber, 1977). Thus, the increase in $S(T_\parallel)$ observed after adding Ca^{2+} to labeled platelets might possibly be due to the environment of the probe becoming more polar, rather than to an alteration in the flexibility of the probe. Control experiments, in which Ca^{2+} was added to purified platelet plasma membranes labeled with a low I(12,3) probe concentration, indicated that $\Delta S(T_\parallel)$, $\Delta S(T_\perp)$ and ΔS exhibited similar increases; moreover, the a'_N values, which are sensitive to the polarity of the membrane, were identical for the control and cation-treated membranes. The most likely interpretation of these results is that the positive $\Delta S(T_\parallel)$ values seen after Ca^{2+} additions to whole platelets reflect a decreased flexibility of the membrane-incorporated probe.

It is important to note that the Ca^{2+}-induced increase in the $S(T_\parallel)$ of I(12,3)-labeled platelets was not affected by the presence of nitroxide radical interactions. Probe titration experiments conducted on intact human platelets indicated that S and $S(T_\perp)$ decreased with increasing probe concentration at sufficiently high probe/lipid ratios, while $S(T_\parallel)$ remained constant. Similar results were obtained in previous I(12,3) probe studies performed on rat liver and heart plasma membrane (Gordon *et al.*, 1978; Sauerheber *et al.*, 1977) and human erythrocytes[1] (Butterfield, Whisnant & Chesnut, 1976). We earlier proposed two distinct physical models to account for the spectral alterations and order parameter effects seen at high probe concentrations (Gordon *et al.*, 1978): (i) The *uniform distribution* model in which the probe is viewed as being homogeneously dispersed throughout the accessible lipid domains for the entire probe concentration range; and (ii) the *patch* model in which the membrane contains only a limited number of binding sites in the lipid matrix that can be occupied by the I(12,3) probe. As the probe concentration increases and these sites become filled, a phase of pure (or, at least, very concentrated) I(12,3) molecules may then co-exist with the endogenous lipids as segregated "patches". Although we cannot presently determine which model more accurately reflects the membrane distribution of I(12,3), the use of high probe concentrations probably does not dramatically perturb the platelet membrane since $S(T_\parallel)$, and the response of $S(T_\parallel)$ to cation additions, is independent of the probe/cell ratio.

The effects that calcium exerts on the fluidity of I(12,3)-labeled platelets are probably due to the binding of Ca^{2+} to the surface membrane. Steiner and Tateishi (1974) earlier examined the total content,

[1] Sauerheber, R.D., Gordon, L.M., Esgate, J.A. *(unpublished observations)*

uptake and distribution of $^{45}Ca^{2+}$ in human platelets. They concluded that platelets contain at least three major Ca^{2+} compartments, one of which is a surface compartment that rapidly equilibrates ($t_{1/2}=4$ min) with extracellular calcium. In agreement with these studies, Robblee and Shepro (1976) employed a lanthanum washout procedure to identify $^{45}Ca^{2+}$ that was initially bound to the surface of calf platelets. With 1 mM calcium in the medium, the cell surface sites exhibited a rapid Ca^{2+}-uptake that reached steady-state within 1–2 min; moreover, the plasma membrane sites were saturable with extracellular Ca^{2+} concentrations and were also highly exchangeable. Our observation that 3.6 mM $CaCl_2$ decreased the platelet fluidity after an 8-min incubation suggests that Ca^{2+} may mediate its effect by interacting with the rapidly-exchangeable surface compartment. Steiner and Tateishi (1974) also studied the distribution of Ca^{2+} among plasma membrane components. Extraction of isolated membranes, which had earlier been incubated with $^{45}Ca^{2+}$, indicated that 21% of the $^{45}Ca^{2+}$ was associated with such membrane lipids as sphingomyelin, phosphatidylcholine, phosphatidylserine, and phosphatidylinositol. It is likely, therefore, that the Ca^{2+}-induced decrease in the fluidity of the platelet surface membrane results from the binding of Ca^{2+} to such phospholipids. However, other membrane constituents (e.g., protein and sialic acids) that bind Ca^{2+} might conceivably be involved in the cation-dependent effects on the fluidity.

We also report that La^{3+} (1 mM) decreases the fluidity of labeled platelets at 37 °C. Additions of La^{3+} have previously been observed to rigidize such I(12,3)-labeled biological membranes as rat heart plasma membranes (Gordon *et al.*, 1978), rat brain synaptosomal membranes (Uyesaka *et al.*, 1976), and *Bacillus subtilis* cytoplasmic membranes (Ehrström *et al.*, 1973). In the case of I(12,3)-labeled platelets, La^{3+} was found to be a more potent agent in decreasing the lipid fluidity than Ca^{2+}. Specifically, the effects of 1 mM $LaCl_3$ or 3.6 mM $CaCl_2$ on the $S(T_{\|})$ of platelets at 37 °C could be mimiced by lowering the temperature by 4 or 2 °C. One possibility that should be explored concerns whether La^{3+} decreases the membrane fluidity by binding to Ca^{2+}-platelet plasma membrane binding sites. La^{3+} has been widely employed as a probe of Ca^{2+}-membrane binding sites, in part because it associates with these sites less reversibly than does Ca^{2+}. The strong binding of La^{3+} to negatively-charged, Ca^{2+}-membrane sites would be expected since the hydrated radius of La^{3+} (3.1 Å) is similar to that of Ca^{2+} (2.8 Å) (Nayler & Harris, 1976). The study of Robblee and Shepro (1976), which employed a La^{3+} wash to displace $^{45}Ca^{2+}$ from the platelet surface membrane, suggests that La^{3+} and Ca^{2+} may each lower the lipid fluidity by binding to the same plasma membrane sites. Additional studies must, however, be performed to determine whether the Ca^{2+} and La^{3+} effects on the fluidity of I(12,3)-labeled platelets are competitive.

The decrease in the platelet membrane fluidity induced by Ca^{2+} or La^{3+} additions may be compared with earlier studies performed on I(12,3)-labeled rat heart plasma membranes. La^{3+} and Ca^{2+} each increased the order parameter of heart membranes at 37 °C, probably by binding to low affinity sites (Gordon *et al.*, 1978). Similar to the results obtained with I(12,3)-labeled platelets, La^{3+} was found to be more effective in lowering the heart membrane fluidity than were equimolar Ca^{2+} concentrations. La^{3+} may exert "supercalcium" effects by binding with high affinity to the Ca^{2+}-membrane sites of biological tissues (Weiss, 1974; Mikkelsen, 1976). It should, however, also be noted that addition of a given Ca^{2+} or La^{3+} concentration orders heart plasma membranes more than that of platelets at 37 °C. The differential response of the fluidities of the heart and platelet membranes to cation additions may be a reflection of differences in lipid composition and/or cation-binding properties. Previous investigations by Pang and Miller (1978) indicated that altering the cholesterol levels in model membranes modulated the response of the lipid fluidity to various perturbants. Perhaps the relatively high *C/Pl* ratio of human platelets blunts the ordering effects of cation additions. Another, possibly related, explanation is that platelet membranes are simply more rigid than heart plasma membranes and are therefore more resistant to additional ordering. However, further studies are required to determine whether higher Ca^{2+} or La^{3+} concentrations will produce larger fluidity effects. Although low and high affinity sites on rat heart plasma membranes were saturated with 2.2 mM $CaCl_2$, Steiner and Tateishi (1974) reported that Ca^{2+} binding to isolated platelet plasma membranes became saturated only when the medium Ca^{2+} concentration reached 10 mM.

Our results indicate that either lowering the temperature below 37 °C or adding Ca^{2+} (or La^{3+}) to I(12,3)-labeled platelets at 37 °C decreased the flexibility of the membrane-incorporated probe. Nevertheless, it should not be assumed that all the lipid structural alterations induced by changing the temperature are identical to those brought about by the addition of Ca^{2+} or La^{3+}. For example, Ca^{2+} causes the formation of domain structure in 1:1 dioleoyl lecithin/dioleoyl phosphatidic acid vesicles (Hartmann *et al.*, 1977) and also induces a lateral separation of phosphatidylserine molecules in mixtures of phosphatidylserine and spin-labeled lecithin (Ohnishi &

Ito, 1974). Such lipid structural rearrangements may well occur in I(12,3)-labeled platelets as a result of Ca^{2+} (or La^{3+}) binding to constituent phospholipids, and it is not particularly likely that temperature reduction would similarly segregate membrane lipids.

One important question concerns whether the spectral alterations induced by Ca^{2+} or La^{3+} additions to labeled platelets are simply due to the specific binding of the cation with the I(12,3) probe. Although this question cannot be answered rigorously at present, there is some evidence to rule out certain modes of cation-probe interactions. It is unlikely that Ca^{2+} or La^{3+} complex with two or more spin probes since these agents do not increase probe-probe interactions, even at high probe loading in platelets where a significant proportion of I(12,3) must be in relatively close contact. Moreover, Ca^{2+} or La^{3+} probably do not bind to individual I(12,3) probe molecules. Here, the binding of Ca^{2+} or La^{3+} to a single spin probe might be expected to neutralize the carboxylic acid group, which would not necessarily lead to a decreased flexibility of the probe; lowering the pH of I(12,3)-labeled lecithin dispersions, for example, loosens the anchoring of the label to the polar membrane surface through protonation of the carboxylic group, thereby permitting the probe to partition into the more fluid hydrophobic interior of the lipid membrane (Barratt & Laggner, 1974). We cannot, however, exclude the possibility that the cations might complex the spin probe to either endogenous phospholipids or protein, although these special cases are not viewed as likely.

The present study is of particular importance since it demonstrates for the first time that extracellular Ca^{2+} or La^{3+} decrease the fluidity of an intact cell. All previous examinations of the effects of Ca^{2+} or La^{3+} on the fluidities of I(12,3)-labeled biological membranes were performed with isolated membranes and not whole cells (*see* above). The plasma membranes of intact mammalian cells are exposed to a well-defined Ca^{2+} gradient, in which the intracellular concentration is generally 10^2–10^5 lower than the extracellular fluid levels (Rasmussen & Goodman, 1977). Therefore, the effects exerted by mM Ca^{2+} on the fluidity of whole platelets *in vitro* are highly significant since this system would be expected to more closely approximate *in vivo* Ca^{2+}-platelet interactions than would *in vitro* studies with purified plasma membranes.

Ca^{2+} and La^{3+} Effects on Platelet Fluidity and Function

The effects that calcium exerts on the lipid fluidity may be important in maintaining the structural and functional integrity of the surface membrane of circulating, unstimulated platelets. The platelet plasma membrane provides a selective permeability barrier for certain substrates, interacts with various agents that stimulate (or inhibit) platelet responses, and contains enzymatic activities involved in platelet homeostasis. It is possible that various platelet membrane-associated functions might be influenced by the lipid structure and/or fluidity, perhaps similarly to that noted in other model and biological membranes. For example, the lipid fluidity may affect such processes as the transport of ions and glucose (Lee, 1975; Jain & White, 1977; Lacko, Wittke & Geck, 1973; Kandutsch, Chen & Heiniger, 1978; Amatruda & Finch, 1979), the binding of certain hormones with plasma membranes of responsive tissues (Kahn, 1976; Mehdi *et al.*, 1977), and the activities of Mg^{2+}- and Na^+, K^+-ATPase (Kimelberg, 1977), and hormone-sensitive adenylyl cyclase (Houslay *et al.*, 1976*a*, *b*). Thus, the binding of Ca^{2+} to platelets might affect a variety of plasma membrane functions by decreasing the lipid fluidity.

Ca^{2+}-dependent alterations in the plasma membrane fluidity may also be involved in the response of platelets to external stimuli. Extracellular calcium has been reported to be necessary for the platelet release reaction as well as the aggregation and adhesion responses of stimulated platelets (Hellem, 1960; Cronberg & Caen, 1970; Heptinstall, 1976; Grinnel, 1978). Moreover, a profound cellular redistribution of calcium is believed to occur when platelets are stimulated by aggregating agents (Lüscher & Massini, 1975; Rasmussen & Goodman, 1977). Any calcium translocations due to platelet activation that affect surface compartment Ca^{2+} might simultaneously alter the plasma membrane fluidity. Therefore, addition of the aggregating agents thrombin, ADP and epinephrine might decrease the platelet fluidity, since these agents have been found to enhance the binding of $^{45}Ca^{2+}$ to the plasma membrane of calf platelets (Robblee & Shepro, 1976). Fluctuations in the fluidity of the surface membrane and/or levels of calcium bound to membrane sites could profoundly influence the fusion of intracellular granules with the platelet plasma membrane, which occurs during stimulus-secretion coupling. Both the lipid fluidity and divalent cations have been suggested to play important roles in membrane fusion (Poste & Allison, 1973).

There is convincing experimental evidence to suggest that adenylyl cyclase (AC), an important, Ca^{2+}-sensitive plasma membrane activity (Vigdahl *et al.*, 1969; Hepp, Edel & Wieland, 1970; Tada *et al.*, 1975; Rodan & Feinstein, 1976), is influenced by the physical state of membrane lipids (Brivio-Haugland *et al.*, 1976; Houslay *et al.*, 1976*a*, *b*; Dipple & Houslay,

1978; Klein, Moore & Pastan, 1978). Factors that affect the activity of platelet AC are of particular interest, since the enzyme product cAMP is a potent inhibitor of platelet aggregation (Haslam, 1975; Salzman *et al.*, 1977); for example, the platelet inhibitor prostaglandin E_1 (PGE_1) activates AC while the aggregating agents ADP and epinephrine inhibit PGE_1-stimulated AC (Mills, 1975). Under conditions that dramatically increase the microviscosity of platelet plasma membranes, Sinha, Shattil and Colman (1977) found that the incorporation of cholesterol into intact platelets effectively prevented the PGE_1-stimulation of AC. It should be noted that mM Ca^{2+} has been reported to decrease both basal and PGE_1-stimulated AC activity (Vigdahl *et al.*, 1969; Rodan & Feinstein, 1976). Although a direct interaction of Ca^{2+} with AC may be responsible for these effects, another possibility is that Ca^{2+}-mediated decreases in the membrane fluidity inhibit PGE_1-stimulated AC.

The La^{3+}-induced rigidization of the platelet membrane may be responsible in part for the effects that this cation exerts on platelet functional processes. Although lanthanum has been reported to inhibit platelet aggregation [caused by ADP (Wörner & Brossmer, 1976)] and thrombin-induced release of serotonin (Robblee & Shepro, 1976), recent studies indicate that La^{3+} itself (1 mM) causes platelet aggregation and serotonin release from washed human platelets, possibly through interaction of the cation with the platelet surface (Wörner & Brossmer, 1976). The La^{3+}-induced aggregation of platelets reported here was found to be inhibited by adenosine (0.03 M). Thus, lanthanum appears to act like other aggregating agents such as ADP, epinephrine, and thrombin, which presumably interact with binding sites on the surface membrane and exert their actions without penetrating the intact cells (Lüscher & Massini, 1975; Nachman, 1975). It must be emphasized that aggregation induced by these agents must differ in certain respects from that caused by lanthanum. Although ADP, epinephrine, and thrombin appear to increase the influx of calcium and the membrane binding of calcium (Robblee & Shepro, 1976), La^{3+} has frequently been found to inhibit the transmembrane fluxes of calcium and to displace calcium from surface membrane sites (Weiss, 1974; Mikkelsen, 1976; Robblee & Shepro, 1976). In any event, these results also suggest, as discussed above, that a relatively rigid membrane may favor platelet aggregation.

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