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EVIDENCE FOR CONSTRAINED LIPID MOBILITY IN THE ERYTHROCYTE GHOST

A SPIN LABEL STUDY

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SUMMARY

Our data show that the ESR spectrum of 5-nitroxide stearate bound to erythrocyte membranes varies with the amount of label bound and suggest that, at high binding, a significant proportion of label molecules lie within $\approx 15 \cdot 10^{-8}$ cm; this gives rise to spin-exchange (ambient temperature) and dipole-dipole interactions. We find that these spectral manifestations due to label clustering can be abolished by reduction of pH and the conjoint action of lysolecithin and trypsin, although both perturbations increase 5-nitroxide stearate binding. Both perturbations are known to mobilize intramembranous particles by modifying or extracting some membrane proteins. We accordingly suggest that the lipids and proteins of erythrocyte membranes exist in a relatively fixed mosaic, and that the mobility of both components is restricted by some membrane-associated protein framework.

INTRODUCTION

One can estimate the lateral mobility of membrane lipids using nitroxide-labelled lipid analogues. When these probes lie within $\lesssim 15 \cdot 10^{-8}$ cm of each other they interact via their magnetic dipoles and by spin-exchange [1–4]. Both processes cause deviations from the ESR spectra obtained with non-interacting nitroxides. This fact has been used to measure the distance between steroid spin labels in phosphatide multibilayers [5], as well as to estimate the rate of lateral lipid diffusion in membranes of *Escherichia coli* [6], *Acholeplasma laidlawii* [7] and sarcoplasmic reticulum [8].

These ESR approaches give few direct clues as to the mobility of membrane proteins, but such information can be obtained by freeze-fracture electron microscopy. This technique reveals that the apolar cores of most biomembranes contain numerous

Abbreviation: 5-nitroxide stearate, 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolidinyloxyl.

uniformly distributed particles $\approx 85 \cdot 10^{-8}$ cm in average diameter, probably representing proteins penetrating into or through the membrane [9]. In some biomembranes, such as the membranes of *Acholeplasma laidlawii* [10, 11] and *E. coli* [12] and the nuclear membranes of *Tetrahymena pyriformis* [13, 14] the intramembranous particles appear to be freely mobile in the membrane plane. This is not normally the case in the plasma membranes of *Tetrahymena pyriformis* [13, 14] or in erythrocyte ghosts. However, exposure of isolated membranes from human erythrocytes to low pH induces severe but reversible aggregation of the intramembranous particles [15, 16]. This pH effect, not seen in intact cells [9], appears to be related to the partial extraction of the erythrocyte membrane protein, spectrin [16]. The conjoint action of lysolecithin and trypsin also causes massive aggregation of the particles in sheep erythrocytes [17], but this is irreversible. Trypsinization following complement-induced and melittin-induced erythrocyte lysis, produces the same particle aggregation [17]. Lysolecithin, trypsin or complement alone do not alter membrane particle distribution.

These data raise the possibility that the structure of the erythrocyte membrane normally restricts the lateral mobility of proteins, and perhaps lipids, in the membrane plane. We have accordingly searched for evidence of spin exchange and dipolar interaction in erythrocyte ghosts, binding large proportions of 5-nitroxide stearate. We here present evidence for such interactions and show that they depend in a predictable fashion on probe binding and membrane state.

EXPERIMENTAL

Chemicals

We obtained 2-(3-carboxypropyl)-2-tridecyl-4, 4-dimethyl-3-oxazolidinyl oxyl (5-nitroxide stearate) from Syva Corporation (Palo Alto, Calif.). Egg lecithin, lysolecithin (Lipid Products, Southnutfield, U.K.) and trypsin (Nutritional Biochemical Corporation, Cleveland, Ohio) were used as supplied. Fat-free bovine serum albumin was obtained from Sigma Biochemicals (St. Louis, Mo.). Other chemicals were of the highest purity available commercially. Hemoglobin-free erythrocyte ghosts were prepared as in ref. 18, using fresh heparinized human blood.

Spin-labelling

We used aqueous solutions of 5-nitroxide stearate for labelling the erythrocyte ghosts. For this, we dissolved 1 mg of 5-nitroxide stearate in 0.05 ml methanol and then added 5 ml of 5 mM phosphate (pH 7.4). After vigorous shaking, the solution was centrifuged at $3.3 \cdot 10^5$ $g \cdot$ min (Spinco L2-65B Ultracentrifuge, rotor SW 56). The electron spin resonance (ESR) spectra of the supernatant fluids and of the uncentrifuged solutions were characteristic of free 5-nitroxide stearate in aqueous solution. Erythrocyte ghosts (typically 5 mg protein measured fluorimetrically, as in ref. 18) suspended in 1 ml 5 mM phosphate (pH 7.4) were then combined with 2 ml of spin-label solution adjusted to give a 5-nitroxide stearate : membrane protein ratio of 0.1–0.2 μmol/mg. After 20 min at 20 °C the erythrocyte spin label suspension was divided into aliquots for further study. For low temperature spectra the ghosts were labelled at ambient temperature prior to ESR spectroscopy.

Liposomes

We prepared egg lecithin/cholesterol/5-nitroxide stearate (5 mg; 1.5 mg; 1 mg) liposomes in 5 mM phosphate (pH 7.4) as in [19]. We also prepared liposomes of cholesterol/egg lecithin (4 : 6 molar ratio) without 5-nitroxide stearate and labelled these by adding 5-nitroxide stearate in aqueous solution, as in the case of erythrocyte ghosts.

Treatment of ghosts with trypsin$\pm$lysolecithin was exactly as in [17], but after spin labelling. A Varian E-9 spectrometer (9.5 GHz, 100 kHz field modulation) was used to record the spectra at room temperature and at -160 °C.

RESULTS

1. ESR spectra at ambient temperature

We have reported previously [20] that 5-nitroxide stearate partitions between membrane and aqueous solution. We calculated the approximate amount of 5-nitroxide stearate taken up by the membranes from the intensities of the high field line due to free spin-label, with and without membrane under various conditions. We found that, at an overall 5-nitroxide stearate : membrane ratio of 0.2 μmol/mg protein, about 80 % of the spin probe binds to unmodified membranes at pH 7.4; the rest remains in the buffer. This estimate is not absolute, due to the mixed spectral contributions of free 5-nitroxide stearate and bound label.

Fig. 1A depicts the ESR spectra of ghost equilibrated with 5-nitroxide stearate (0.2 μmol/mg protein). Two types of spectral contribution can be resolved: (a) that due to free spin label in solution (splitting = 15.7$\pm$0.5 G), (b) that due to bound 5-nitroxide strearate with restricted motion (maximum splitting 61$\pm$0.5 G); this is accompanied by much greater peak-to-peak distance, ΔH, of the central band (8.2 G), than that observed in liposomes. Erythrocyte ghosts equilibrated with 0.12 μmol/mg protein, take up about 90 % of the spin label. Under these conditions maximum splitting is 56 G and ΔH is 5 G.

Liposomes containing 1 mg 5-nitroxide stearate per 5 mg of lecithin yield a ΔH value of 5 G; no signal due to free 5-nitroxide stearate is detected. However, when egg lecithin/cholesterol liposomes are labelled by partitioning with dissolved 5-nitroxide stearate (0.15 mM) by the procedure used for erythrocyte ghosts, ΔH is 4.3 G and a small signal due to free spin label is observed. The maximum splitting is 56 G in both cases.

One explanation for the greater width of the central peak in ghosts compared with liposomes might be that 5-nitroxide stearate occurs in a clustered distribution in the biomembranes. As shown in [3] when spin label pairs lie within an average distance of $15\text{–}20 \cdot 10^{-8}$ cm, spin exchange can occur and this can cause an increased ΔH of the central band. However, restricted anisotropic motion can also increase ΔH. For example, the value of ΔH for 5-nitroxide stearate bound to bovine serum albumin is 7.0 G at a 1 : 5 molar ratio of 5-nitroxide stearate : protein, where the protein's three binding sites are not saturated. With < 1 molecule 5-nitroxide stearate per protein molecule no probe interactions should occur; this suggestion is supported by our low temperature studies.

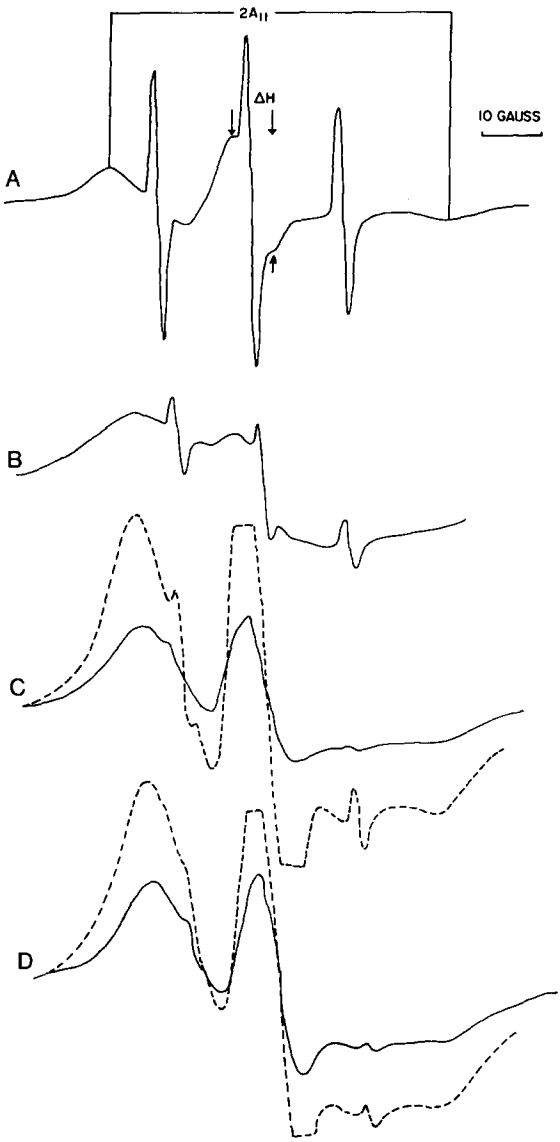

Fig. 1. ESR spectra of 5-nitroxide stearate in erythrocyte ghosts ± trypsin and lysolecithin. (A) ghosts; (B) ghosts + trypsin; (C) ghosts + lysolecithin; (D) ghosts + trypsin + lysolecithin. Dotted curves in C and D obtained at 10 × higher amplification. Procedural details in text.

2. Effect of pH change

Lowering the pH from 7.4 to 6.2 decreases ΔH from 8.2 G to 7.2 G, reduces maximum hyperfine splitting from 61.0 G to 58 G, but causes a further uptake of the 5-nitroxide stearate free in solution at pH 7.4 (Fig. 2). When the pH is further dropped from 6.2 to 5.3, ΔH diminishes to 6.2 G, the maximum hyperfine splitting decreases slightly to 56 G and there is additional uptake of 5-nitroxide stearate. Such pH

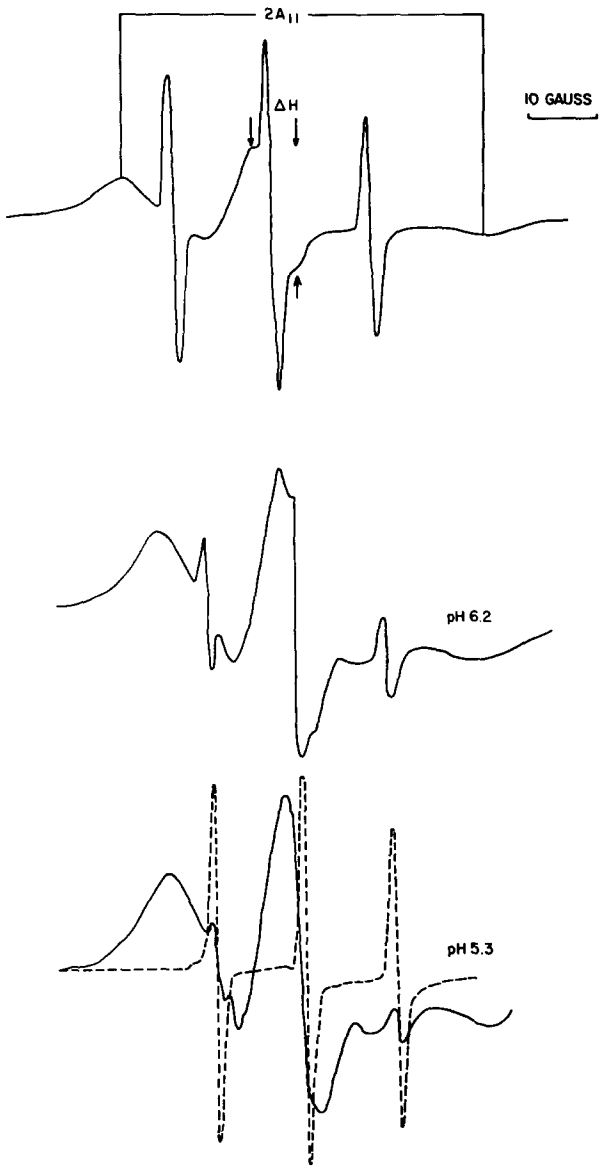

Fig. 2. ESR spectra of 5-nitroxide stearate in erythrocyte ghosts at pH 7.4, 6.2 and 5.3. The dotted curve (in lower part) is that of free 5-nitroxide stearate at pH 5.3.

changes do not affect the spectra obtained with lecithin/cholesterol liposomes, and bovine serum albumin exhibits the same ΔH and maximum splitting between pH 10 and pH 2 (7.0 G and 64.0 G respectively).

3. Action of trypsin and/or lysolecithin

A typical ESR spectrum recorded 30 min after treating spin-labelled ghosts,

equilibrated with 0.1 μmol 5-nitroxide stearate/mg protein with 100 μg trypsin/mg ghost protein, is presented in Fig. 1B. The following is evident (a) $\approx$ 60 % of the spin-label unbound in control membranes has gone into the membrane, (b) the maximum splitting reduces only marginally ($\approx$ 1 G) and ΔH stays at approx. 8.0 G. The addition of lysolecithin alone (100 μg/mg protein) effects the transfer of virtually all of the free 5-nitroxide stearate to the membrane. The maximum splitting is reduced to 56 G, but ΔH decreases only slightly to 7.5 G. The combined effect of trypsin and lysolecithin is to produce transfer of virtually all free spin-label to the membrane and to reduce maximum splitting to 54 G, significantly ΔH decreases sharply to 6.2 G.

The addition to 60 μg trypsin/mg lipid of egg lecithin/cholesterol liposomes reduces the maximum splitting from 56.25$\pm$0.5 G to 50.0$\pm$0.5 G but does not change in the intensity of free signal; the basis for this effect is not known. The addition of lysolecithin decreases maximum splitting from 56 G to 51 G but does not cause 5-nitroxide stearate uptake. The combined effect of trypsin and lysolecithin on

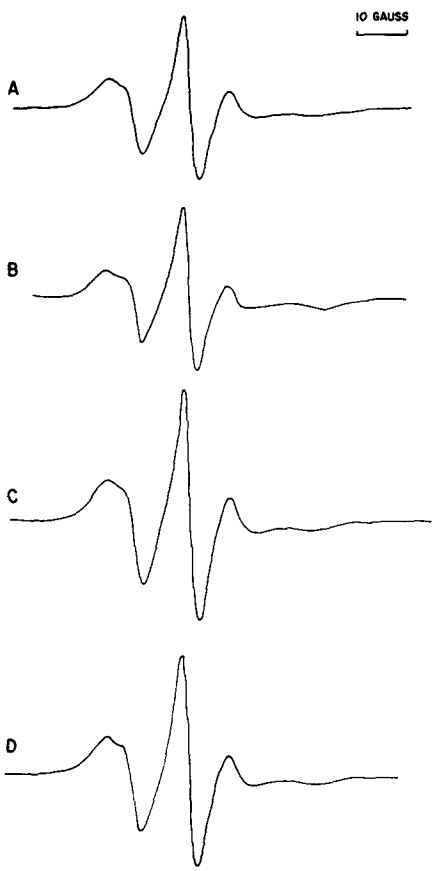

Fig. 3. ESR spectra of 5-nitroxide stearate in egg lecithin + cholesterol liposomes $\pm$ trypsin and lysolecithin. (A) egg lecithin/cholesterol liposomes; (B) egg lecithin/cholesterol liposomes + trypsin; (C) egg lecithin/cholesterol liposomes + lysolecithin; (D) egg lecithin/cholesterol liposomes + trypsin + lysolecithin.

egg lecithin/cholesterol liposomes is similar to that of either agent alone. Maximum splitting is 50 G with no change in ΔH or the proportion of free spin probe.

4. Low temperature spectra

Because one cannot interpret changes in ΔH unambiguously under conditions where the molecular motion of bound 5-nitroxide stearate might be changed by a perturbation, we have recorded ESR measurements also at −160 °C. Under these circumstances molecular motion becomes negligible; i.e. the probes (and their ligands) are frozen in position. The spectra recorded then do not depend on probe-tumbling or probe-diffusion, and the contribution of spin exchange between closely located labels is slight at low temperatures. The low temperature spectra reflect (a) the static orientational distributions of the label molecules and (b), when spin labels lie within $\approx 15 \cdot 10^{-8}$ cm, interactions between their magnetic moments. Dipolar interactions will contribute in three spectral areas: in the region of the central peak and far from the central peak in both the low-field and high-field directions. Ideally these contributions resolve into distinct bands.

The low temperature ESR spectra of erythrocyte ghosts at 0.2 μmol 5-nitroxide stearate per mg protein are compared with those of 0.2 mol 5-nitroxide stearate per mol bovine serum albumin in Fig. 4. The curves exhibit the general features of rigid glass spectra [21]. 5-Nitroxide stearate/bovine serum albumin yields the same ΔH value for the central peak, 12–13 G, at 5-nitroxide stearate : protein molar ratios ranging between 1 : 5 and 5 : 1. This value fits that reported for 16-nitroxide stearate in lecithin at −180 °C [21]. The ghost spectra differ from those of 5-nitroxide stearate/albumin and 16-nitroxide stearate/lecithin in two important ways: (a) The ΔH value for the central peak is large, i.e. 17–18 G, compared to 12–13 G. (b) The low field peak is much broader than in the case of 5-nitroxide stearate/bovine serum albumin (Fig. 4) or 16-nitroxide stearate/lecithin [21]. The intensity and rigid-glass character of the ghost spectra indicates that the 5-nitroxide stearate remains membrane-bound and the proportion of free 5-nitroxide stearate does not contribute significantly to the

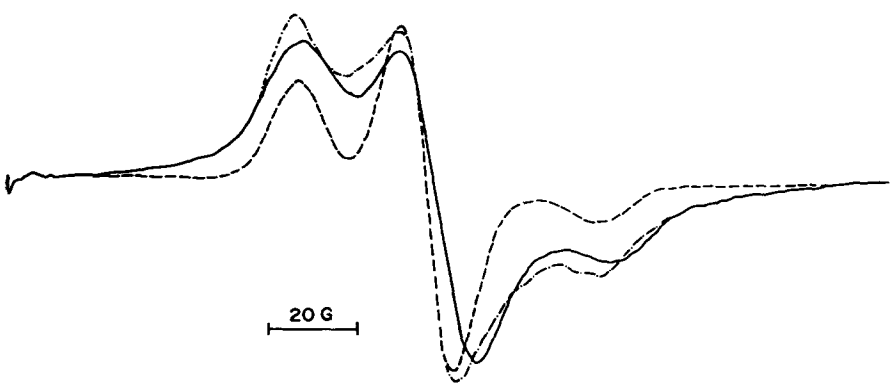

Fig. 4. ESR spectra at −160 °C of 5-nitroxide stearate in the presence of bovine serum albumin, erythrocyte ghost±trypsin and lysolecithin; ——, erythrocyte ghost+5-nitroxide stearate; - - - - - erythrocyte ghost, treated with trypsin+lysolecithin; - - -, bovine serum albumin+5-nitroxide stearate (1 : 1 molar ratio).

spectrum. However, when we equilibrate ghosts with lower 5-nitroxide stearate levels (0.12 μmol 5-nitroxide stearate per mg protein) ΔH drops to 12–13 G and the low field region of the spectrum also becomes indistinguishable from that of 5-nitroxide stearate/bovine serum albumin. Under these conditions 5-nitroxide stearate uptake is about 0.10 μmol/mg.

Low temperature ESR spectra of erythrocyte ghosts labelled at high 5-nitroxide stearate : protein ratios (0.2 μmol/mg), but treated with trypsin and lysolecithin become indistinguishable from those recorded at -160 °C with 5 nitroxide stearate/bovine serum albumin or ghosts at low 5-nitroxide stearate : protein ratios.

DISCUSSION

Spin-labelled probes such as 5-nitroxide stearate do not associate covalently with biomembranes; rather they distribute between their membrane-binding sites and the suspension medium according to simple thermodynamic principles. Accordingly, perturbation of the membranes, whether chemical or physical, might alter not only the character of the membrane binding sites, but also the distribution of the label between the membrane- and bulk-phases. Such a redistribution process cannot be reliably detected when membranes are washed to remove excess spin label, as is common, because the label remaining membrane-associated, will be that bound to sites of an affinity high enough ($K_A \geqslant 10^5$) to hinder detection of free label by ESR spectroscopy.

In the set of experiments presented here, we have equilibrated erythrocyte ghosts with 5-nitroxide stearate dissolved in buffer solutions at various 5-nitroxide stearate : membrane ratios, and have recorded the ESR spectra of the suspensions at ambient temperature and at -160 °C to determine label uptake, peak-to-peak width, ΔH, of the central band and maximum hyperfine splitting.

We find that at pH 7.4, both ΔH and maximum splitting increase with the amount of 5-nitroxide stearate bound by erythrocyte ghosts. Two possibilities exist: (a) equilibration of the membranes with high concentrations of 5-nitroxide stearate leads to the label-binding at immobilized sites, (b) increased binding leads to clustering of 5-nitroxide stearate molecules and a concomitant increase of ΔH (and splitting) due to spin-exchange [3]. We favor the second possibility on the basis of the low temperature spectra, where the contribution of molecular motion is essentially eliminated. Here the ghost exposed to 0.2 μmol 5-nitroxide stearate per mg protein show a ΔH of 17–18 G compared with 12–13 G for ghosts treated with 0.12 μmol/mg. The value of 12–13 G is comparable to that found with 5-nitroxide stearate/bovine serum albumin and reported for 16-nitroxide stearate/lecithin.

The peak-to-peak distance (ΔH) of the central band in the ESR spectra of nitroxide spin probes is given by [6]

$$\Delta H = \Delta H_0 + \Delta H_{dip} + \Delta H_{ex}$$

where ΔH_0 is the line width at "magnetically dilute" concentrations of the spin probe, ΔH_{dip} is the line broadening due to magnetic dipolar interactions among spin label pairs, and ΔH_{ex} is contributed by spin-spin exchange.

ΔH_0 depends on the motion of the spin probe which complicates interpretation of changes in ΔH at ambient temperatures. ΔH_{ex} depends on the exchange frequency

(W_{ex}) which relates to the distance, d, between the two exchanging spins as in ref. 3

$$W_{ex} = Ae^{-\alpha d}$$

where A and α are constants which can be determined experimentally. Accordingly, W_{ex} decreases rapidly with increasing d. W_{ex}, hence ΔH_{ex}, becomes small at low temperatures. ΔH_{dip} is not readily detected at usual temperatures but can be detected at low temperatures. The line-pair separation, due to magnetic dipolar interaction, varies inversely with the cube of the lateral separation of the labels.

At −160 °C all bands widen and splitting increases also. However, ΔH for erythrocyte ghosts exposed to 0.2 μmol 5-nitroxide stearate/mg protein lies at 17–18 G, much in excess of the value of 12–13 G found in bovine serum albumin or lecithin labelled with small amounts of 16-nitroxide stearate. This is not due to free 5-nitroxide stearate which contributes negligibly under the conditions employed. Ghosts treated with only 0.12 μmol 5-nitroxide stearate/mg protein also show a ΔH of 12–13 G. The 18 G value can thus be attributed to dipole-dipole interaction; i.e. ΔH_{dip} is $\approx$ 5 G at −160 °C. Accordingly the high ΔH of 8.2 G at high loading and ambient temperature must arise, at least in part, from spin exchange.

What might be the basis for the interactions between 5-nitroxide stearate molecules seen at high loading? To obtain some clues we have evaluated the effects of two modes of membrane perturbation, namely pH reduction and the separate or combined actions of trypsin and lysolecithin. Both approaches induce drastic structural changes within the apolar cores of the membranes, which can be detected by freeze-etch electron microscopy [15–17]. These membrane perturbations reflect in the ESR spectra of 5-nitroxide stearate in three ways, namely: (a) changes in the signal arising from free 5-nitroxide stearate in the buffer due to alterations in the distribution of 5-nitroxide stearate between membrane and medium, (b) alterations in the maximum hyperfine splitting of bound 5-nitroxide stearate due to changes at the binding sites and (c) changes in the peak-to-peak distance, ΔH, of the central peak, which, we reason, arise from altered interactions between 5-nitroxide stearate molecules.

The perturbations indicate that the amount of 5-nitroxide stearate which associates with erythrocyte ghosts depends on the membrane state. Thus, reduction of pH to $\approx$ 5.3 progressively increases the amount of membrane-bound 5-nitroxide stearate yet reduces maximum hyperfine splitting as well as ΔH. Two possibilities exist: (a) The pH change alters membrane proteins, exposing new binding sites. However, if anything, this should increase not decrease splitting as well as ΔH; also, in bovine serum albumin neither parameter changes significantly between pH 10 and pH 2. (b) pH reduction alters the general membrane organization to allow increased molecular motion and to accommodate more 5-nitroxide stearate, probably in association with membrane lipids. We favor this view since the pH 5.3 spectra exhibit identical maximum hyperfine splitting as observed in cholesterol/lecithin liposomes. This argument also fits Pinto da Silva's electron microscopic observations [15] of enhanced lateral mobility of intramembranous particles at pH $\approx$ 5.

Turning to the action of trypsin and lysolecithin, we note that both induce 5-nitroxide stearate uptake. Presumably different mechanisms are involved, since lysolecithin, but not trypsinization, significantly reduces maximal splitting. Importantly, neither agent alone markedly affects ΔH but conjointly they reduce ΔH to 6.2 G (ambient temperature), the same value found at pH 5.3. Also, the conjoint

action of trypsin and lysolecithin reduces ΔH_{-160° to 12–13 G, the value for non-interacting nitroxides.

We relate these facts to our previous observation [17] that neither lysolecithin nor trypsin alone alters the distribution of intramembranous particles, whereas their conjoint action causes particle aggregation. This correlation suggests that the behavior of the spin probe reported here may reflect the behavior of some membrane lipids rather than only the special physical properties of the label.

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