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Evidence which Suggests the Existence of Lipid Regions Discrete from Those of the Protein in Mitochondrial and Red Blood Cell Membranes

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Summary. The action of phospholipase C(EC 3.1.4.3) on erythrocyte ghosts and submitochondrial particles results in a marked change in the molecular structure of the phospholipid regions of the membrane but in no change in the environments (i.e., structure) of the sulfydryl and tyrosyl residues of the membrane proteins as determined by electron spin resonance "spin label" studies. These results hold true whether the membrane is one containing a relatively high proportion of cholesterol (erythrocyte) or one containing very little cholesterol (mitochondrial). The relevance of these findings to current membrane models is discussed.

The application of physical methods, such as circular dichroism (CD) [14, 23], nuclear magnetic resonance (NMR) [10], and infra-red spectroscopy [16, 24] have led to new proposals concerning the structure of membranes. Experiments [6] involving the use of the enzyme phospholipase C (EC 3.1.4.3), which hydrolyzes phospholipids to diglycerides and phosphorylated amines, have suggested that such enzyme treatment does not affect the helical conformation of the membrane proteins, as measured by CD, but rather results in a substantial fraction of the cleaved phospholipids becoming mobile, which in turn gives rise to strong proton resonances observable by NMR. These results suggest that a substantial proportion of the phospholipid can possess an altered structure without affecting the helical conformation of the membrane proteins.

The importance of these observations concerning membrane structure is obvious, and we have continued to pursue this line of research by employing another physical technique – "spin labelling". Our approach relies on the introduction of a small, stable, paramagnetic nitroxide radial into the

membrane. The environment of the nitroxide determines the shape and size of the electron spin resonance (ESR) signal. The technique has already been used to yield structural information about the organization of membranes [8] and of membrane proteins [18]. In order to determine whether differences exist in the lipid regions between a membrane containing cholesterol and one which does not, mitochondrial membranes prepared from beef heart (low cholesterol) were compared to plasma membranes prepared from red blood cells (0.22 mg cholesterol per mg membrane protein).

Materials and Methods

Red blood cell (RBC) membranes were prepared from fresh human blood of various blood types according to the method of Dodge, Mitchell and Hanahan [5]; submitochondrial particles (SMP), fragments of the inner mitochondrial membrane, were prepared by the method of Racker [17], except that a buffer of 0.25 M sucrose, 2 mM ATP, and 2 mM Tris-tricine, pH 7.4, was employed instead of sucrose-pyrophosphate. Phospholipase C from *Clostridium welchii* (Sigma Chemical Co., St. Louis, Mo.) was homogenized in 2 mM KCL, 2 mM $CaCl_2$, and 5 mM imidazole, pH 7.4 (or 5 mM Tris-HCl, pH 7.4), heated to 60 °C for 5 min, and finally centrifuged at $100{,}000 \times g$ before use. This enzyme preparation possessed no proteolytic activity as determined by the negligible release of ninhydrin-positive material, loss of total protein, and changes in the polyacrylamide gel electrophoresis patterns (performed according to Lenard [12]) after enzyme treatment. Generally, 0.4 units of phospholipase C per mg membrane protein was used, and this resulted in the liberation of 60 to 70% of the total membrane phosphorus, determined by the method of Bartlett [2]. (One unit of phospholipase C liberated 1 μmole of phosphorus per minute from egg lecithin at 37 °C.)

Spin labels (Fig. 1a and b) were bought from Synvar Associates (Palo Alto, Calif.). The spin label based on N-acetyl-imidazole (Fig. 1c) was prepared as described by Barratt, Dodd and Chapman [1]. The steroid spin label (Fig. 2a) was prepared according to Keana, Keana and Beethan [11], and the 12-nitroxide stearate spin label (Fig. 2b) according to the method of Waggoner *et al.* [22]. The 5-keto stearic acid was prepared [9], and then converted to the nitroxide [22] (Fig. 2c). The nitroxide stearates required further

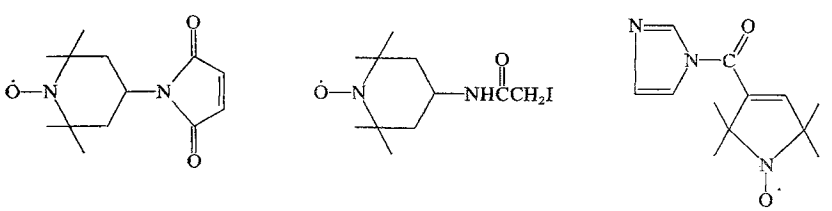
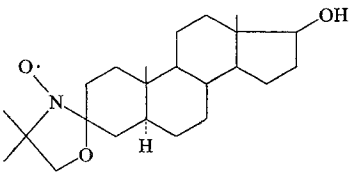
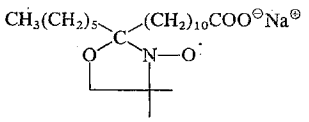


Fig. 1a–c. The molecular formula of the protein spin labels. (a) 4-maleimido-2,2,6,6-tetramethyl piperidinooxyl (NEM spin label). (b) 4-(2-iodoacetamido)-2,2,6,6-tetramethyl piperidinooxyl (iodoacetamide spin label). (c) N-(2,2,5,5-tetramethyl-3-carbonylpyrroline-1-oxyl) imidazole



a)



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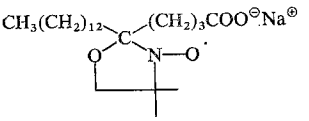

c)

Fig. 2a–c. The steroid and fatty-acid spin labels employed in this study. (a) 17-β-hydroxyl-4′,4′-dimethylspiro-(5-α-androstane-3,2′-oxazolidin)-3′-yloxyl. (b) 2-(10-carboxydecyl)-2-hexyl-4,4-dimethyl-3-oxazolidinyloxyl. (c) 2-(3-carboxypyopyl)-4,4-dimethyl-2-tridecyl-3-oxazolidinyloxyl

purification on a 40 × 2 cm silica gel G(60–120 mesh) column with hexane as the solvent followed by thin-layer chromatography on silica gel G with hexane as developer followed by hexane:ether (7:3 v/v), followed again by hexane development. Labelling of the membranes by the protein spin labels, shown in Fig. 1, was performed by mixing (at 23 °C for 3 hr) a filtered solution of the spin label and the membrane in the ratio of 1:4 w/w (label to membrane protein) in the buffer used for storing the respective membranes. The fatty-acid and steroid spin labels were absorbed into a 5% w/v solution of fatty acid-free BSA (Calbiochem) in the membrane buffer, and then exchanged overnight at 4 °C from the albumin-spin label complex into the respective membranes. (The ratio of label−albumin complex to membrane protein−was 1:7 v/w.) The excess protein or lipid spin label was removed by repeated centrifugation of the membranes in their respective buffers. The ESR spectra were obtained with a Jeolco spectrometer (JES-ME-1X), run at 9.5 mHz. The membrane protein was determined by the method of Lowry *et al.* [15].

Results

Protein labels, 1(a) and 1(b), based on N-ethyl maleimide and iodoacetate, respectively, tend to label specifically membrane sulfydryl groups. Their ESR spectra, when bound to membrane protein, remained unchanged when those membranes were treated with phospholipase C (Figs. 3b & c and 4a & b). It should, however, be noted that the RBC membrane control, which had been incubated in the 2 mM KCl − 2 mM $CaCl_2$ − 5 mM imidazole pH 7.4 buffer at 37 °C for 20 min, showed some change (due to the Ca^{++}-

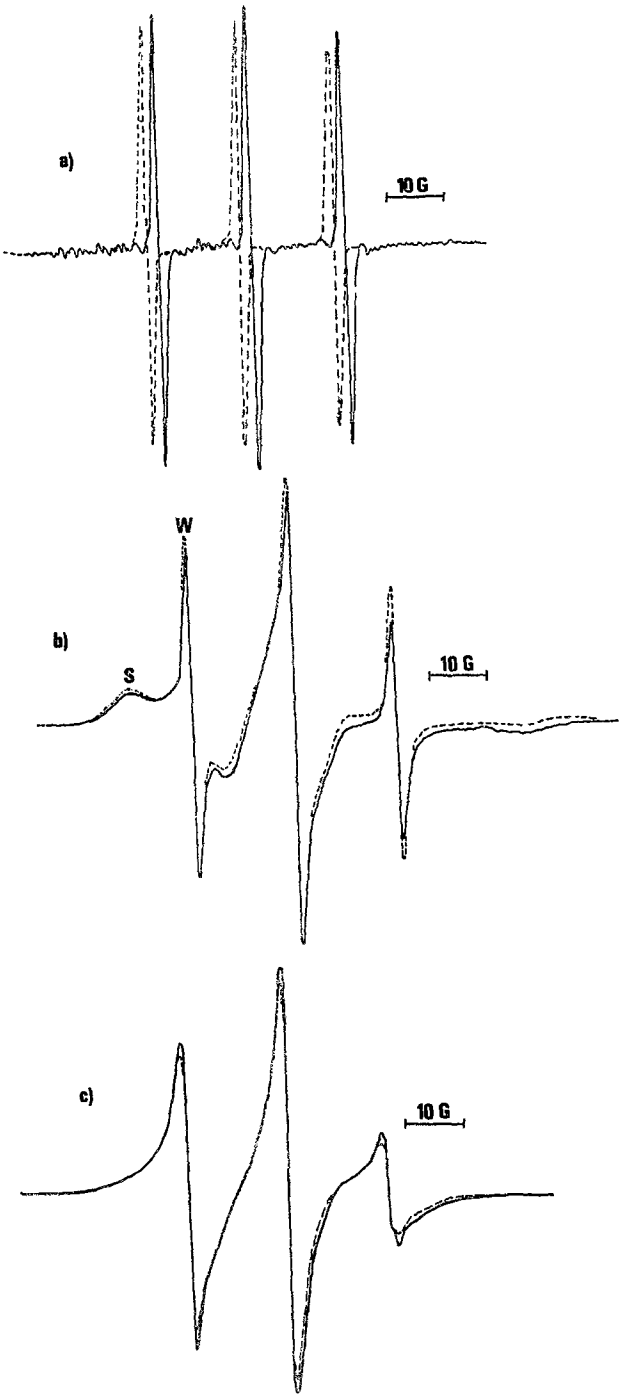

Fig. 3a–c. The ESR spectrum obtained with the protein spin labels and RBC membranes. (a) The tyrosine spin label before (———) and after (------) enzyme treatment (displaced 2 G for clarity). (b) The N-ethyl maleimide spin label before (———) and after (------) phospholipase C treatment. (c) The iodoacetamide spin-labelled membranes before (———) and after (------) enzyme treatment

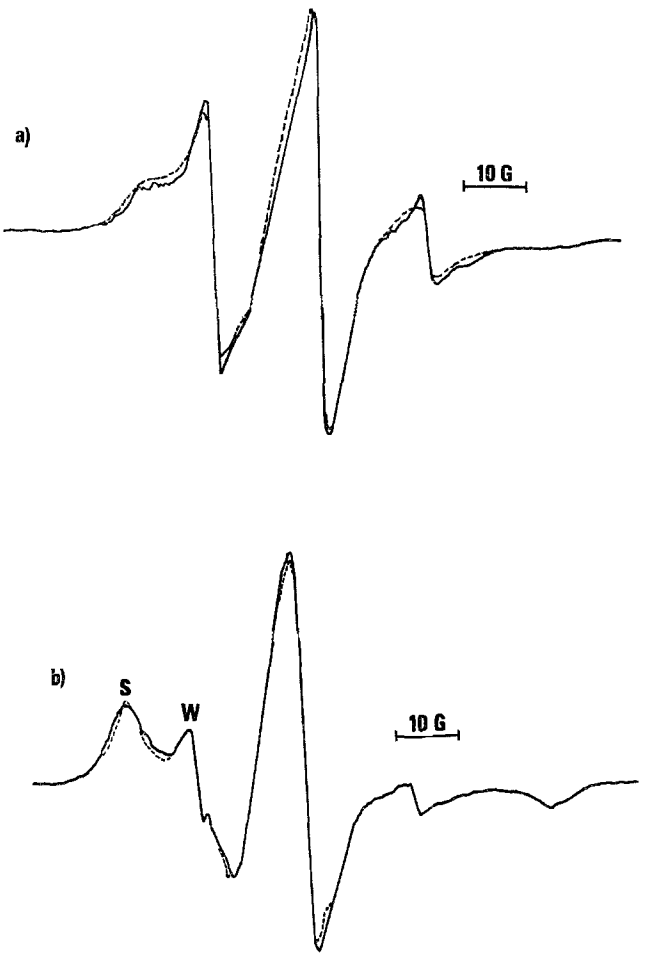

Fig. 4a and b. The ESR spectrum obtained with the protein spin labels and SMP. (a) The iodoacetamide spin label before (———) and after (-------) enzyme treatment. (b) N-ethyl maleimide spin labelled membrane before (———) and after (-------) enzyme treatment

Tris or Ca^{++}-imidazole medium) when compared to those membranes labelled in phosphate buffer. The phospholipase C-treated RBC membranes, however, resulted in a spectrum identical to that obtained with membranes prepared and labelled in 7 mM phosphate buffer (pH 7.4) (Fig. 3b). Similarly, the tyrosine-specific spin label, which appeared to label tyrosine residues only in a hydrophilic environment, i.e., on the exterior of the membrane surface, remained unchanged after enzyme treatment (Fig. 3a). These results showed that not only does the helical content of the membrane proteins remain unchanged, as shown by previous CD measurements [6], but that

the environments of the sulfydryl and tyrosine residues remained unaffected by extensive phospholipase C treatment.

The fatty-acid spin-label molecules possess a charged group at one end, and are therefore assumed to reside in the membrane lipid regions with the charged group at or near the membrane-solvent interface. These labels showed that molecular changes in the conformation of the membrane lipids occurred on phospholipase C treatment. In Figs. 5a and 6a are shown, respectively, the spectra of the 12-nitroxide stearate in RBC membranes and SMP and the spectra obtained after enzyme treatment of these membranes. The changes in the spectra show an increase in the ratio of H/L of 70 to 80%. This reflects an increasing fluidity of the fatty acid chains produced by cleavage of the charged phospholipid headgroups. That is, the spectra, after enzyme treatment, resemble more those produced by the label in a highly mobile state than in a solid or highly immobile state [21].

The spectra of the 5-nitroxide stearate, in which the paramagnetic nitroxide group probably resides nearer the exterior of the membrane and is, therefore, closer to the ionic headgroups of the phospholipids, resulted in a spectrum in untreated membranes (Figs. 5b and 6b), possessing a lower H/L ratio than the 12-nitroxide. This reflected a decreasing mobility of the nitroxide group, when placed near the polar end of the stearic acid molecule. The hyperfine splitting (T) was greater with RBC membranes (55.8 G) than with the SMP (57.8 G) indicating that the nitroxide environment in the cholesterol-containing membrane possesses a lower mobility. Enzyme treatment results in the splittings (T) observed in Figs. 5 and 6 between the outer doublet (ε) increasing 1 to 2 gaus with RBC membranes and 2 to 3 gaus with SMP membranes, reflecting an increased fluidity of the molecules surrounding the 5-nitroxide group. A comparison of the spectra obtained with the enzyme-treated membranes (presented in Figs. 5b and 6b) also shows two other important features. The increase in the hyperfine splitting (ΔT) and the increase in the amplitude of θ relative to that of ε on phospholipase C treatment are greater with the SMP ($\Delta T \simeq 2.5$ G, $\Delta(\theta/\varepsilon) = 17\%$) than with the RBC ($\Delta T \simeq 1.5$ G, $\Delta(\theta/\varepsilon) = 11\%$) membranes.

The steroid spin label, based on the 5 α-androstan-3one-17 βol (*see* Fig. 2a), possesses a hydroxyl group instead of the hydrocarbon side chain present in cholesterol and has the nitroxide ring situated at the 3 position of the steroid nucleus. The spectra obtained with this label in RBC membranes remained unchanged on phospholipase C treatment (*see* Fig. 5c). This could mean that either (a) the position where the nitroxide is situated in the membrane is not near the phospholipids which have been digested by the enzyme (30% of the phospholipid phosphorus is not liberated by

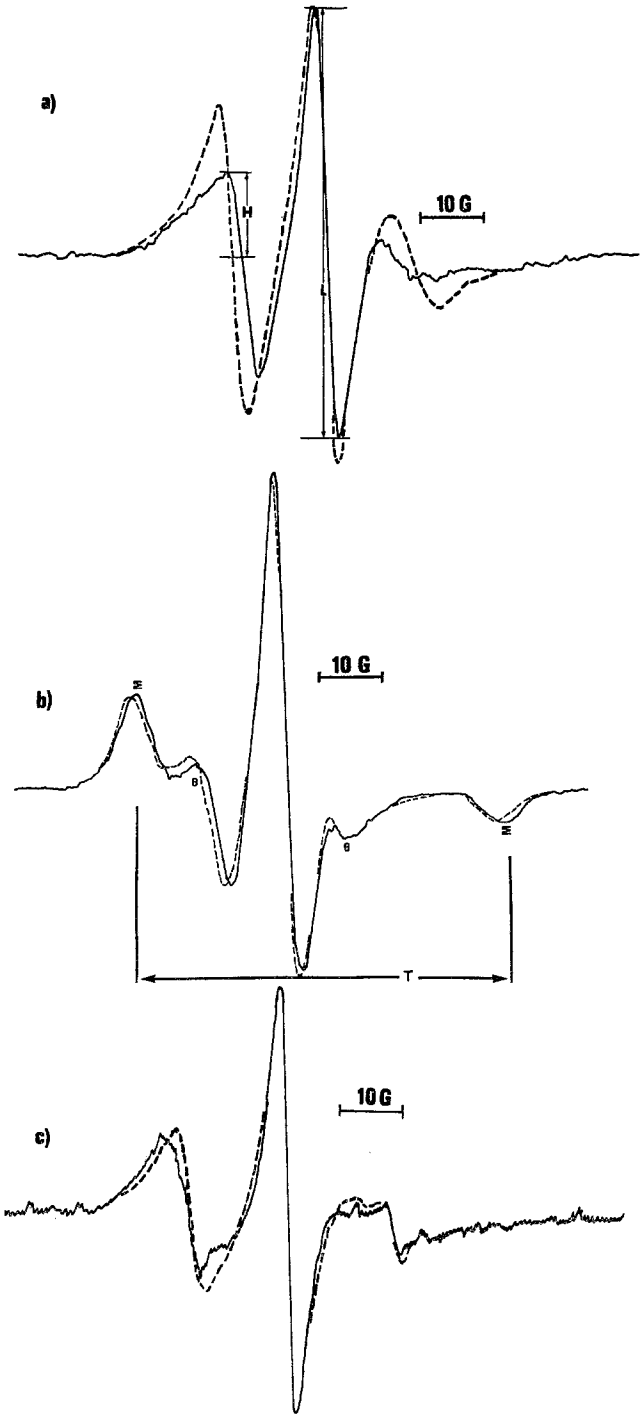

Fig. 5a–c. The ESR spectra obtained with RBC membranes labelled with fatty acid and steroid spin labels. (a) The 12-nitroxide stearate before (———) and after (-------) treatment with phospholipase C. (b) The 5-nitroxide stearate before (———) and after (-------) treatment with phospholipase C. (c) The androstane spin label before (———) and after (-------) enzyme treatment

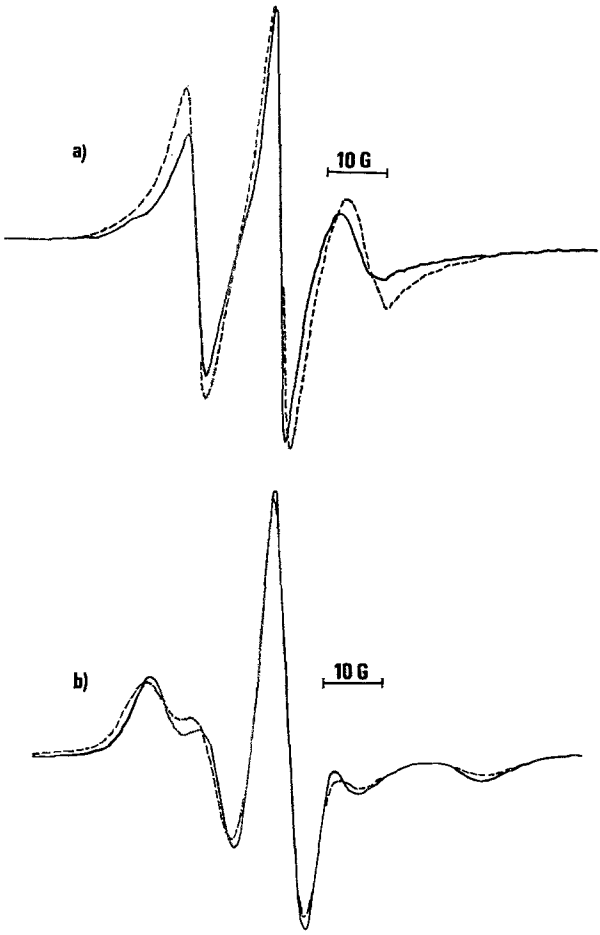

Fig. 6a and b. The ESR spectra obtained with SMP membrane labelled with the fatty acid spin labels. (a) The 12-nitroxide stearate before (———) and after (------) treatment with phospholipase C. (b) The 5-nitroxide stearate before (———) and after (-------) treatment with phospholipase C

enzyme treatment), or (b) the nitroxide group in this molecule is situated in the central membrane core, which is unaffected by the removal of the ionic headgroups of the phospholipids on the membrane exterior.

Discussion

The increased mobility observed in the membrane lipid regions after enzyme treatment substantiate earlier studies employing PMR techniques [6]. However, the spin label studies provide more information as to the nature of these structural changes.

Analysis of ESR spectra [8] and evidence based on the relative rates of nitroxide reduction by ascorbate (H. Simpkins, *unpublished observations*) suggest that the 12-nitroxide is situated deeper in the lipid regions of the membrane than the 5-nitroxide. Enzyme treatment appears to affect the fatty acids over their whole length as the ESR spectra show that the environments of both nitroxides increase in mobility. However, even in the case of the 12-nitroxide stearate, the spectra resulting from the enzyme-treated membrane does not reflect that of a nitroxide in a completely mobile environment. Also, the environments of the 5-nitroxide stearate in both membranes become somewhat more fluid after enzyme treatment, but the outer hyperfine doublet (ε) can still be resolved showing that the nitroxide's motion is still quite severely restricted and anisotropic. These results are in agreement with the previous PMR data which showed that not all the available protons contributed to the PMR spectrum after enzyme treatment. However, it is now shown that the mobility of the fatty acid chain after enzyme treatment increases more as the distance from the charged end group is increased. Also the mitochondrial membranes possess a more mobile lipid structure than the RBC membranes (hyperfine splitting T is smaller), and the spectral changes produced by enzyme treatment also appear greater with the mitochondrial membrane. These observations can be explained by the stabilization of the fatty-acid chains of the phospholipids and diglycerides by cholesterol as suggested by Dervichian [4].

Spin labels based on N-acetyl imidazole, N-ethyl maleimide, and iodoacetamide were employed to determine if any changes occur in the environments of the tyrosine or sulfydryl groups of the membrane proteins after phospholipase C action. The specificity of the N-ethyl maleimide and iodoacetamide spin labels for membrane proteins was checked in various ways. Firstly, a maximum of 10% of the label can be extracted with chloroform: methanol (2:1 v/v). Secondly, N-ethyl maleimide, *p*-chloromercuribenzoate and iodoacetamide block 80 to 100% of the labelling possible with these two spin labels [18, 19]. Separation of disaggregated RBC membrane proteins on polyacrylamide gels labelled with ^{14}C-iodoacetamide results in all of the radioactivity being recovered in the gels, and proteins labelled with ^{14}C-N-ethyl maleimide result in 80 to 90% of the total radioactivity being recovered [13] in the protein bands. These results with polyacrylamide gels suggest that N-ethyl maleimide and iodoacetamide themselves predominantly react with the membrane proteins. As these compounds block the reaction of their respective spin label derivatives with the membrane proteins, it seems reasonable to assume that N-ethyl maleimide and iodoacetamide spin labels react predominantly with the membrane sulfydryl groups. The tyrosine

spin label has also been shown to bind preferentially to poly-L-tyrosine rather than to poly-L-lysine [1], but it is difficult to determine if a small proportion of the lysine residues in the membrane proteins are labelled with this spin label.

That the spin labels are very sensitive to changes in protein conformation is shown by the following experimental observations. The spectra of the NEM and iodoacetamide spin-labelled RBC membranes are grossly ffected by anions, such as Tris and oxalate. Detergents such as sodium dodlecyl sulfate (0.2%) show a marked change in the N-ethyl maleimide spin label spectrum [19] (increasing the mobile component of the spectrum); lysolecithin at very low concentrations (0.05%) perturbs the spectra but in the opposite sense. Our results and others [7] also indicate that the addition of cations, such as 0.1 M Na^+, and changes in pH [3] produce large changes in the N-ethyl maleimide spin-labelled ghost spectrum. These protein spin labels are therefore sensitive indicators of local conformational changes in the membrane proteins, and, as shown in Figs. 3 and 4, their resonance spectra do not change after phospholipase C treatment. This provides further evidence that the membrane protein structure remains unaffected. A criticism that can be levied is that the proteins and lipids labelled *after* enzyme treatment may exhibit spectral changes because of different membrane regions becoming labelled preferentially as to those labelled *before* treatment. Any ESR signal changes observed will therefore reflect changes in the availability of certain membrane regions to the label; however, membranes labelled *before* treatment with phospholipase C and then enzyme treated result in the same spectra as those labelled *after* enzyme treatment.

The lipid and protein spin-label data therefore provide additional evidence to that previously presented [6] that phospholipase C treatment produces gross structural changes in the lipid regions of the membrane, but none in the protein regions. The presence or absence of cholesterol does not influence these conclusions. The existence of a substantial proportion of the lipid being structurally discrete from that of the protein is therefore suggested.

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