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HYDROPHOBIC BINDING SITES IN BOVINE SERUM ALBUMIN AND ERYTHROCYTE GHOST PROTEINS

STUDY BY SPIN-LABELLING, PARAMAGNETIC FLUORESCENCE QUENCHING AND CHEMICAL MODIFICATION

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SUMMARY

1. The binding of 5- and 16-nitroxide stearates to bovine serum albumin and erythrocyte ghosts has been measured by paramagnetic quenching of protein fluorescence

2 Bovine serum albumin exhibits saturable binding only, while erythrocyte ghosts show both saturable and unsaturable components

3 When bovine serum albumin saturated with 5- or 16-nitroxide stearate is reacted with *o*-phthaldehyde, a lipophilic dialdehyde, all the bound spin-label becomes displaced into aqueous solution

4 *o*-Phthaldehyde produces a similar effect with erythrocyte ghosts equilibrated at similar level of spin label However, about 5 % of the spin label remains membrane bound

5 Sulphydryl blockers, such as *p*-chloromercuribenzoate, reduce the action of *o*-phthaldehyde on ghosts by about 70 %, but have no effect in the case of serum albumin

6 The reaction of *o*-phthaldehyde with the phosphatidylethanolamine in erythrocyte membrane lipid does not produce displacement of nitroxide stearates

7. We suggest that certain erythrocyte membrane proteins are surrounded by a shell of closely associated lipid, which is perturbed with certain protein modifications We further point out analogies between lipid protein interactions in erythrocyte membranes and serum albumin

Abbreviations PCMB, *p*-chloromercuribenzoic acid, 5-nitroxide stearate, 2-(3-carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolidinyloxyl, 16-nitroxide stearate, 2-(14-carboxy-tetradecyl)-2-ethyl-4,4-dimethyl-3-oxazolidinyloxyl, maleimide spin label, 4-maleimido-2,2,6,6,-tetramethylpiperidinoxyl, isothiocyanate spin label, 4-isothiocyanato-2,2,6,6,-tetramethylpiperidinoxyl

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INTRODUCTION

In earlier publications on possible structural devices allowing proteins to penetrate into, or through the apolar cores of biomembranes, we pointed to the likelihood of close apolar associations between phosphatide hydrocarbon chains and the lipophilic perimeters of membrane proteins [1, 2] Accordingly, penetrating proteins should be encased by phosphatide shells, whose structuring depends upon protein architecture. In these boundary layers, the phosphatidyl acyl chains immediately adjacent to the protein would assume configurations which allow optimal topologic apposition with apolar protein surfaces. With increasing distance from the protein perimeters, lipid configuration would become less and less influenced by the protein Recent studies with fluorescent [3] and spin-label [4, 5] probes, show that certain membrane proteins are surrounded by an "immobilized" lipid boundary layer, as suggested

The postulated apolar protein perimeters could come about through a highly unusual amino acid composition or through tertiary and/or quaternary structuring which, in contrast to the situation common for proteins in aqueous solution [6], interiorizes polar residues and externalizes hydrophobic amino acid side chains This arrangement might be favored on energetic grounds in the membrane case, where the "solvent" for many amino acid residues is apolar membrane lipid, creating a situation analogous to the formation of micelles with interiorized polar groups, upon transfer of lipid amphiphiles into organic solvents [7]. The postulated lipid–protein interaction would be expected to exhibit a number of parallels to lipid binding by water-soluble proteins with apolar pockets, e.g. serum albumin, and such parallels have already been documented for lysolecithin binding by erythrocyte membranes and bovine serum albumin [8, 9].

The binding of fatty acids, detergents and other hydrocarbons by serum albumins have been very well studied and the binding site(s) extensively characterized [10-15]. Of particular interest is the fact that aldehyde treatment of serum albumins impairs binding of certain amphiphilic anions.

We have accordingly studied the effect of aldehyde modifications of erythrocyte membrane proteins upon certain lipid–protein interactions in erythrocyte ghosts, using bovine serum albumin for comparison. We report in particular upon the effect of the lipophilic dialdehyde, *o*-phthaladehyde, which interferes with the binding of 5- and 16-nitroxide stearates to erythrocyte ghosts and bovine serum albumin. We further demonstrate the utility of paramagnetic quenching of protein fluorescence in the study of lipid–protein interactions.

EXPERIMENTAL

Chemicals

Chemicals of highest available commercial purity were used. *o*-Phthaldehyde, *β*-mercaptoethanol, ethylmaleimide, *p*-chloromercuribenzoic acid (PCMB) and fat free bovine serum albumin were obtained from Sigma (St. Louis, Mo.). 2-(3-Carboxypropyl)-2-tridecyl-4,4-dimethyl-3-oxazolidinyloxyl (5-nitroxide stearate) and 2-(14-carboxytetradecyl)-2-ethyl-4,4-dimethyl-3-oxazoli-dinyloxyl (16 nitroxide stearate), 4-maleimido-2,2,6,6,-tetramethylpiperidinoxyl (MSL) and 4-isothiocyanato-2,2,6,6,-

tetramethylpiperidinoxyl (ISL) from Syvar (Palo Alto, Calif) and glyoxal from Metheson Coleman and Bell (Norwood, Ohio)

Hemoglobin-free erythrocyte ghosts were prepared as in [16], using fresh, heparinized human blood (O/RH^+)

Analyses

Protein was measured fluorometrically as in [16], ghost lipids were extracted as in [17] and washed membrane lipids [18] were analysed by thin-layer chromatography on Silica gel H (Merck, Darmstadt) using chloroform–methanol–water (300:190:45, by vol) as developer [19], and specific membrane phosphatides were identified by reference to authentic lipids (phosphatidylethanolamine, lysophosphatidylethanolamine, lecithin, lysolecithin, sphingomyelin, Lipid Products, South Nutfield, Great Britain), run simultaneously

Spin labelling

We have developed a simple means for labelling ghosts with 5-nitroxide stearate and 16-nitroxide stearate, using aqueous solutions of these nitroxides. For this we add 5 mg of 5-nitroxide stearate or 16-nitroxide stearate to 2 ml 50 mM phosphate buffer (pH 7.4) shake vigorously and, after 1 h centrifuge at $3\,3 \cdot 10^5$ g · min. (Spinco L2-65B Ultracentrifuge, Rotor SW56). We repeated this step 3 times With both 5-nitroxide stearate and 16-nitroxide stearate, the supernatant fluids exhibit three symmetrically spaced, sharp ESR lines of equal height, typical of freely tumbling nitroxides

After kinetic studies showed that the binding of nitroxide stearates is essentially complete within 20 min at room temperature, we established conditions for ghost labelling as follows· Different amounts of ghosts (0 01–5.0 mg protein) were equilibrated in 0 5 ml 50 mM phosphate buffer (pH 7 4), saturated with 5-nitroxide stearate and 16-nitroxide stearate. After 20 min at room temperature, we centrifuged at $3\,3\ 10^5$ g min and measured the free nitroxide remaining in the supernatant fluid We found that approx 0 1 mg ghost (protein) per ml saturated nitroxide-stearate solution, essentially eliminates all of the spin label from aqueous solution and, unless stated otherwise, all experiments were conducted at these nitroxide/ghost ratios (As we shall show in our paramagnetic-quenching experiments considerably more nitroxide–stearate binds with excess of the spin label). After labelling, ghosts were washed thrice with 50 mM phosphate buffer (pH 7 4) ($3\,3 \cdot 10^5$ g min) to remove excess spin label

To label with MSL and ISL, we dissolve these in 50 mM phosphate buffer (pH 7.4) and react with normal ghosts and membranes treated with *o*-phthaldehyde, at a final proportion of 1 mg label/10 mg ghost protein Excess spin label was removed by washing thrice with 50 mM phosphate buffer (pH 7 4)

Paramagnetic quenching of protein fluorescence

Ghost or bovine serum albumin (0.05–0 10 mg protein in 50 mM phosphate buffer (pH 7.4) up to 50 % satn; $3\,20 \cdot 10^{-4}$ M) were titrated with 5-nitroxide stearate or 16-nitroxide stearate. Tryphophan fluorescence becomes stable within 10 min after changing nitroxide stearate concentration. For both ghosts and bovine serum albumin control measurements were paired with readings on spin-label-containing samples

Because of their negligible light absorption, aqueous solutions of nitroxide

stearates produced no inner filter effects. Moreover, under conditions identical to those employed for ghosts and bovine serum albumin, neither 5-nitroxide stearate nor 16-nitroxide stearate quenches the fluorescence of aqueous solutions of tryptophan.

Treatment with o-phthaldehyde

We proceeded as in ref. 20, adding 0-15 μmoles *o*-phthaldehyde to 1 mg ghost protein (control and spin-labelled ghost) in 100 μl 50 mM phosphate buffer (pH 7.4). After incubating in the dark, at room temperature for 1 h, the cells were washed once with 50 mM phosphate buffer (pH 7 4) (3.3 10^5 g · min).

Treatment with β-mercaptoethanol PCMB and N-ethylmaleimide

In some experiments, β-mercaptoethanol (1 5 · 10 μmoles/ghost protein) was added and the ghosts were incubated at room temperature for 15 min prior to addition of *o*-phthaldehyde, MSL, etc In certain other studies, PCMB or *N*-ethylmaleimide were added, before or after *o*-phthaldehyde, to 5-nitroxide stearate or 16-nitroxide stearate labelled ghosts at final concentrations of 0.6 μmoles PCMB or *N*-ethylmaleimide/mg ghost. PCMB solutions of 35.7 mg/ml in 0.02 M NaOH were used. Ghost suspensions were maintained at pH 8 0 by titrating with 0.02 M HCl during PCMB addition.

Multilayers and liposomes

Phosphatidylethanolamine multibilayers were prepared as in [21]. We also prepared liposomes of lecithin: phosphatidylethanolamine (1.1, w/w) and of total lipids extracted from normal and *o*-phthaldehyde-treated ghosts, by sonicating the vacuum-dried lipids in 50 mM phosphate (approx. 10 %, by wt.) for 20 min. (power step 30 sonic dismembranator, Quigley-Rochester, Rochester, N.Y.).

ESR spectroscopy

ESR spectra were obtained on the modified Varian ESR spectrometer, with X–Y recorder, at the Boston Biomedical Institute, Boston, Mass. Representative spectra from 4–5 experiments are presented.

Laser Raman spectroscopy

Laser Raman Spectra are obtained on a Ramalog IV spectrometer (Spex Industries, Metuchen, N. J.) using an ion–argon laser tuned at 488.0 nm.

Fluorescence measurements

A Perkin–Elmer MPF 3A fluorescence spectrophotometer was employed. Excitation is at 286 nm (slitwidth 4 nm). Emission is measured at 338 nm (slitwidth 5–8 nm).

RESULTS

ESR of 5-nitroxide stearate and 16-nitroxide stearate bound to control ghosts or bovine serum albumin

5-Nitroxide stearate bound to erythrocyte ghost yields fairly immobilized anisotropic ESR spectra (Fig. 1). The maximum hyperfine separation is 56 G. The spectrum has the shape reported earlier [22].

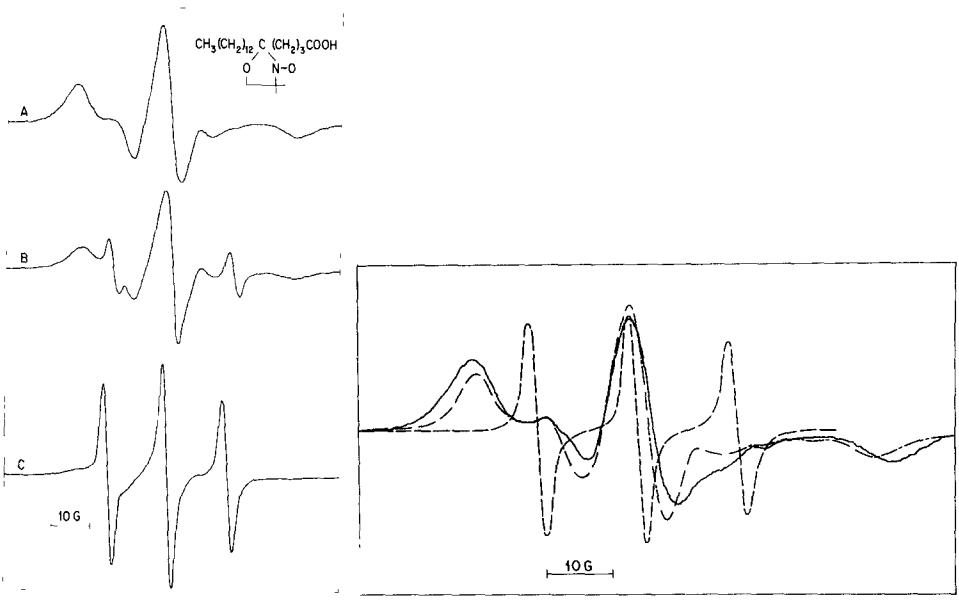


Fig 1 ESR spectrum of 5-nitroxide stearate in the presence of erythrocyte ghosts and varying levels of *o*-phthaldehyde A, no *o*-phthaldehyde, B, 7 5 μmoles *o*-phthaldehyde/mg ghost protein, C, 15 μmoles *o*-phthaldehyde/mg protein Details in text ESR spectra taken approx 1 h after *o*-phthaldehyde treatment

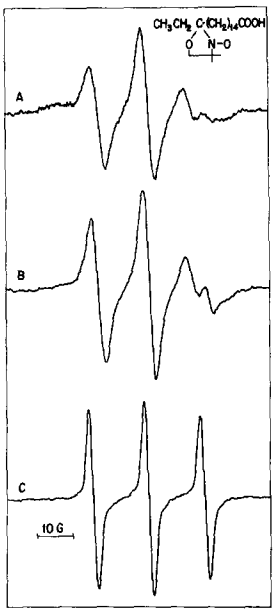
Fig 2 ESR spectra of 5-nitroxide stearate and 16-nitroxide stearate in the presence of bovine serum albumin and of 5-nitroxide stearate in the presence of bovine serum albumin and *o*-phthaldehyde Details in text —, 5-nitroxide stearate, bovine serum albumin - - -, 5-nitroxide stearate, bovine serum albumin, *o*-phthaldehyde, - - -, 16-nitroxide stearate + bovine serum albumin. Spectra taken after approx 1 h

The ESR spectra of 5-nitroxide stearate bound to bovine serum albumin (Fig 2) indicate very high probe immobilization and a maximum hyperfine separation of 64 G 16-Nitroxide stearate when bound to ghosts produces ESR spectra such as shown in Fig 3. Most of the nitroxide exhibits substantial molecular motion However, some of the probes reside in less fluid regions Hence, the 16-nitroxide stearate spectrum is a composite, one due to partially-immobilized molecules and one due to more strongly immobilized 16-nitroxide stearate; this may reflect the presence of two types of apolar binding sites (The outer hyperfine extrema, separated by 47 3 G, arise from the less mobile probes)

The ESR spectrum of 16-nitroxide stearate bound to bovine serum albumin (Fig 2) shows much greater probe immobilization than found in ghosts, with the outer peaks separated by 60 5 G

Effect of o-phthaldehyde

o-Phthaldehyde produces a drastic change in the ESR spectra of 5-nitroxide stearate labelled ghosts. As shown in Fig 1, 7 5 μmoles *o*-phthaldehyde/mg ghost protein, produces ESR spectra, dominated by components arising from "free" spin probes The intensity of the "free" signal increases with *o*-phthaldehyde concentration



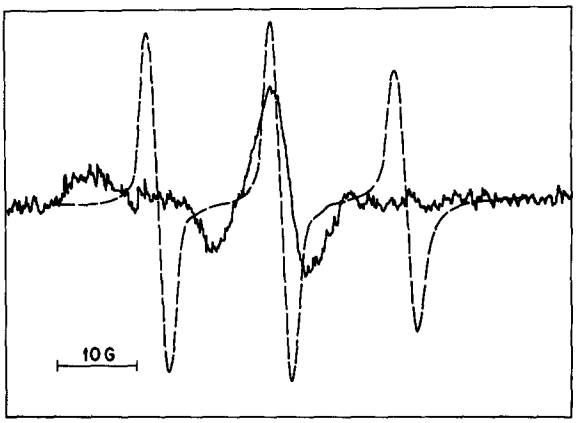


Fig 3 ESR spectra of 16-nitroxide stearate in the presence of ghosts, with and without *o*-phthaldehyde Details in text A, no *o*-phthaldehyde, B, 1 5 μmoles *o*-phthaldehyde/mg ghost protein, C, 15 μmoles/mg protein Spectra taken after approx. 1 h.

Fig 4 ESR spectra of 5-nitroxide stearate in separated suspension medium (- - -) and ghost pellet (—) after *o*-phthaldehyde treatment of ghosts (15 μmoles *o*-phthaldehyde/mg ghost protein) Details in text The spectrum of the pelleted ghosts was recorded at 100 times the gain used for the supernatant

At 15 μmoles *o*-phthaldehyde/mg ghost protein, the "strongly immobilized" component of the ESR spectra has almost disappeared. The "free" component shows three lines, separated by 15.7$\pm$0.3 G, a value typical for the spin label in aqueous solution

When we treat 5-nitroxide stearate labelled ghosts with *o*-phthaldehyde (15 μmoles/mg ghost protein) and centrifuge ($3\,3 \cdot 10^5$ g · min), we obtain supernatants, whose spectra (Fig. 4) are characteristic of free nitroxide. Over 95 % of the probe has been displaced into the aqueous phase. The ESR spectra of the residues show that about 5 % of the probe remains bound in a relatively immobile fashion. *o*-Phthaldehyde (15 μmoles/mg) also displaces all bound 5-nitroxide stearate from bovine serum albumin–5-nitroxide stearate complexes into aqueous solution (Fig.2).

We observe similar effects after we treat 16-nitroxide stearate-labelled ghosts with *o*-phthaldehyde (Fig. 3). With 7.5–15 μmoles *o*-phthaldehyde/mg ghost protein, a highly mobile ESR component emerges, and at 15 μmoles *o*-phthaldehyde/mg ghost protein the 16-nitroxide stearate spectrum is virtually isotropic and typical of the probe dissolved in aqueous media.

Reaction of bovine serum albumin with *o*-phthaldehyde (15 μmoles/mg) produces complete displacement of 16-nitroxide stearate into aqueous solution (Fig. 2)

Effect of sulphydryl reagents

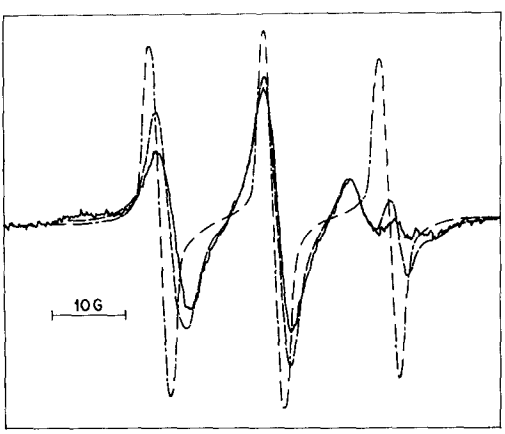
o-Phthaldehyde can form thioesters and/or hemithioacetals with sulphydryl amino acids and/or proteins [20] This is shown by laser–Raman spectroscopy for

β-mercaptoethanol-*o*-phthaldehyde (powder), methanolic *o*-phthaldehyde and β-mercaptoethanol (1·1 ratio, w/w) and methanolic *o*-phthaldehyde and β-mercaptoethanol (1 2 ratio, w/w) The sulphydryl stretching vibrations appear at 2570 cm^{-1} in β-mercaptoethanol and the carbonyl vibrations at 1680 cm^{-1} (*o*-phthaldehyde) These peaks disappear almost completely after reaction with *o*-phthaldehyde A new band appears at 703 cm^{-1} which has been previously assigned due to C–S vibrations [23]

To evaluate whether sulphydryl groups participate in the displacement of 5-nitroxide stearate or 16-nitroxide stearate from ghosts or bovine serum albumin, we have attempted to block this action of *o*-phthaldehyde with PCMB or *N*-ethylmaleimide Neither sulphydryl blocker alone alters the spectra of ghost-bound 5-nitroxide stearate or 16-nitroxide stearate comparably to *o*-phthaldehyde However, PCMB decreases maximum hyperfine splitting by 2 G and displaces a small fraction of 5-nitroxide stearate from ghosts

After blocking the sulphydryl groups of ghost protein with PCMB or with *N*-ethylmaleimide, addition of *o*-phthaldehyde still changes the ESR spectra, producing signals due to "free" nitroxide (Fig 5) However, the change is less pronounced than with *o*-phthaldehyde alone From the ratio of the central peak to the high field peak (due to free spin probe) we calculate a 60–70 % inhibition of the *o*-phthaldehyde effect. However, sulphydryl blockers do not inhibit *o*-phthaldehyde-induced displacement of nitroxide stearate from bovine serum albumin by more than 5 %

MSL reacts preferentially with sulphydryl [24] although interaction with $-NH_2$ has been reported also [25]. When bound to ghosts, MSL yields a composite ESR spectrum. One component, P_1. P_2 (Fig 6) arises from partially immobilized MSL.



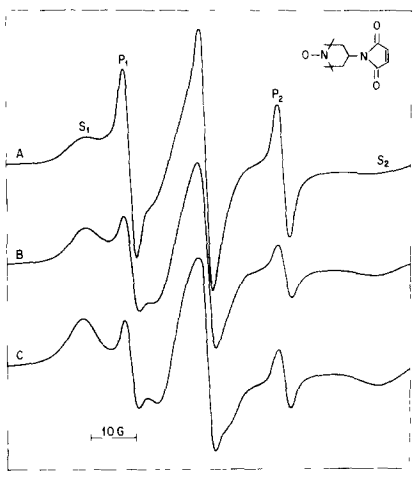


Fig 5 Effect of PCMB on the *o*-phthaldehyde induced displacement of 16-nitroxide stearate bound to ghosts Details in text —, ghosts, 16-nitroxide stearate, - - - -, ghost, 16-nitroxide stearate, *o*-phthaldehyde, - - -, ghosts, 16-nitroxide stearate, *o*-phthaldehyde, PCMB Spectra were taken after 1 h

Fig 6 Effect of *o*-phthaldehyde on the ESR spectra of maleimide spin label bound to erythrocyte ghosts. For details see text A, no *o*-phthaldehyde, B, 5 μmoles *o*-phthaldehyde/mg ghost protein C, 13 μmoles *o*-phthaldehyde /mg protein

the other, S_1, S_2 arises from strongly immobilized MSL. S_1 and S_2 are separated by 60 G Thus, MSL binds to two sites. One could be at the surface (corresponding to the P_1, P_2 peaks) and the other, corresponding to S_1 and S_2, within the membrane [24]. We use the amplitude ratio of the Peaks P_1 and S_1 to evaluate the relative proportions of the two binding sites.

o-Phthaldehyde (<13 μmoles/mg) changes the relative amplitudes of the Peaks P_1 and S_1 (Fig. 6), i.e. the height of P_1 decreases while S_1 increases with increased *o*-phthaldehyde concentration. Table I presents the S_1/P_1 ratios for MSL in the presence of *o*-phthaldehyde and β-mercaptoethanol+*o*-phthaldehyde When we add β-mercaptoethanol before *o*-phthaldehyde, the relative amplitudes of the Peaks P_1 and S_1 remain essentially unchanged

TABLE I

EFFECT OF *o*-PHTHALDEHYDE ON S_1/P_1 PEAK RATIO IN ESR SPECTRA OF MALEIMIDE LABELLED GHOSTS

See text for detail

o-Phthaldehyde (μmoles/mg protein)	S_1/P_1 ratio	β-mercaptoethanol (μmoles/mg protein)	*o*-Phthaldehyde (μmoles/mg protein)	S_1/P_1 ratio
0 0	0 14	15	0.0	0 27
0 3	0 43	15	0 3	0 20
0 75	0 44	15	0.75	0.21
1.5	0 56	15	1 5	0 19
3 0	0 50	15	3 0	0 20

In the other sets of experiments, we labelled the ghosts with ISL prior to *o*-phthaldehyde addition Then the ESR spectra show identical ESR spectra as control membranes. ISL reacts specifically with the amino groups.

Lack of nitroxide-stearate displacement by glyoxal

The dialdehyde, glyoxal, has been shown to perturb the hydrophobic binding site of bovine serum albumin by reaction with arginines [15] However, under the conditions given in [15], glyoxal does not effect any displacement of 5-nitroxide stearate or 16-nitroxide stearate discernable by ESR spectroscopy in either bovine serum albumin or ghosts.

Reaction of arginine with 5-nitroxide stearate and/or o-phthaldehyde

1 h after combining equal volumes of a 7.4 mM solution of L-arginine and a saturated solution of 5-nitroxide stearate (both in 50 mM phosphate buffer (pH 7.4)) we obtain the ESR spectrum given in Fig. 7. Three narrow lines, due to free 5-nitroxide stearate lie superimposed upon on broad band. The latter indicates spin–spin interaction between closely associated probe molecules in arginine–5-nitroxide stearate complexes. Addition of a molar excess of *o*-phthaldehyde abolishes the broad band (Fig. 7), indicating disruption of the arginine–5-nitroxide stearate complexes.

Laser–Raman spectroscopy shows that *o*-phthaldehyde reacts with arginine In these experiments, *o*-phthaldehyde in methanol solution was diffused into crystal-

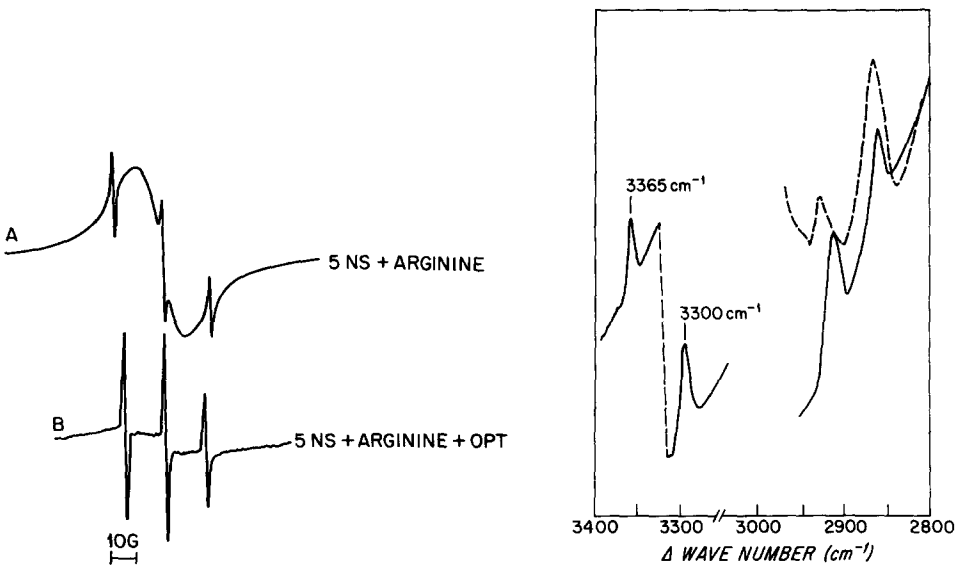


Fig 7 Effect of *o*-phthaldehyde on ESR spectrum of 5-nitroxide stearate in the presence of arginine Experimental details given in text A, arginine, 5-nitroxide stearate, B, arginine, 5-nitroxide stearate, *o*-phthaldehyde Spectra taken approx 1 h after *o*-phthaldehyde treatment

Fig 8 Laser Raman spectra of L-arginine with (- - -) and without (—) *o*-phthaldehyde There is a baseline adjustment between 3300 and 3350 cm^{-1}

line arginine The NH stretching frequencies of arginine lie at 3300 and 3365 cm^{-1} Both disappear upon the binding of *o*-phthaldehyde. Importantly, the ratio of the peak height at 2860 and 2920 cm^{-1} (2920 cm^{-1}/2860 cm^{-1}) changes from 1 5 to 0 27 with and without the presence of *o*-phthaldehyde (Fig 8). The latter two peaks are due to $-CH_3$ and $-CH_2$ stretching modes This suggests that *o*-phthaldehyde binding affects the environment around the $-CH_2$ group of arginine Presently, we are not concerned with the details of *o*-phthaldehyde–arginine reaction

Reaction of o-phthaldehyde with phosphatidylethanolamine

o-Phthaldehyde forms Schiff bases with numerous primary amines, including phosphatidylethanolamine (unpublished results) Reaction with lipid has nothing to do with the effects observed in bovine serum albumin, but might affect binding of 5-nitroxide stearate and 16-nitroxide stearate by erythrocyte ghosts We have accordingly examined the ESR spectra of 5-nitroxide stearate and 16-nitroxide stearate incorporated into liposomes prepared from whole lipids extracted from ghosts treated with 15 μmoles *o*-phthaldehyde/mg protein As shown in Fig 9, these spectra suggest at best a slight decrease (approx 5 %) in the order parameters [26] compared with those of liposomes prepared from the lipids of untreated ghosts There is no indication of "free" nitroxide in the *o*-phthaldehyde liposomes, although thin-layer chromatography indicates that an appreciable proportion (but not all) of the ghost phosphatidylethanolamine has reacted with *o*-phthaldehyde *o*-Phthaldehyde–phosphatidylethanolamine migrates ahead of phosphatidylethanolamine with the solvent system employed (R_F = 0 85 vs 0 75 for phosphatidylethanolamine) Similarly, *o*-phthaldehyde treat-

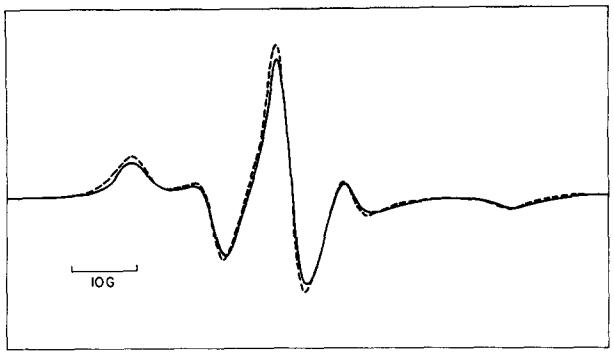


Fig 9 ESR spectrum of 5-nitroxide stearate bound to liposomes prepared from total lipid extracts of untreated ghosts (—) and ghosts previously reacted with *o*-phthaldehyde (- - -) See text for details

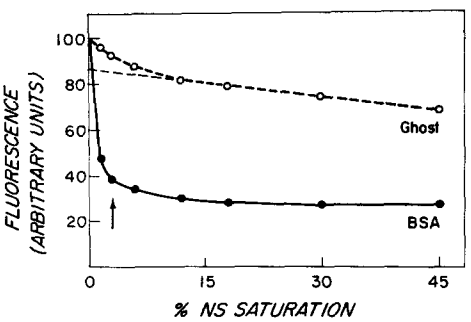


Fig 10. Paramagnetic quenching of tryptophan fluorescence with increasing concentration of 16-nitroxide stearate. Ordinate, fluorescence in arbitrary linear units Abscissa, % saturation with 16-nitroxide stearate, where 100 % = 0 63 mM Curve 1 bovine serum albumin (final concn 30 μg/ml) Curve 2, erythrocyte ghosts (final concn 30 μg/ml) Extrapolation to 0 concentration of 16-nitroxide stearate points out high affinity binding, as do data points in this region Arrow indicates operating range for *o*-phthaldehyde experiments

ment of lecithin–phosphatidylethanolamine multibilayers or liposomes, containing nitroxide stearates, does not lead to nitroxide extrusion, although, it does change the ESR parameters. Thus, although some phosphatidylethanolamine does react when erythrocytes are treated with *o*-phthaldehyde, this cannot account for the observed displacement of 5-nitroxide stearate and 16-nitroxide stearate.

Paramagnetic quenching

We have used paramagnetic quenching of tryptophan fluorescence to provide a measure of nitroxide binding to tryptophan, and/or tyrosine-bearing sites on bovine serum albumin or membrane proteins.

As shown in Fig. 10, even low concentrations of 16-nitroxide stearate (3 % satn· $1.9 \cdot 10^{-5}$ M) produce marked quenching of bovine serum albumin fluorescence. Maximum loss of fluorescence occurs at about 18 % satn

Ghosts behave differently. The quenching effects are less marked than in bovine serum albumin Moreover, after a small, sharp decrement in fluorescence at very low concentrations of 16-nitroxide stearate, fluorescence decreases linearly with increasing

levels of 16-nitroxide stearate in a non-saturable fashion 5-Nitroxide stearate behaves analogously at low concentrations, but its poor solubility has prevented simple exploration of the concentration range where ghost quenching exceeds 20 %

DISCUSSION

How does *o*-phthaldehyde modify the interactions of 5-nitroxide stearate and 16-nitroxide stearate with bovine serum albumin and erythrocyte ghosts and what do our data signify in terms of lipid–protein interactions?

Our ESR spectra, in accordance with previously published data, indicate considerable immobilization of the two spin probes by both bovine serum albumin and ghosts. As anticipated the degree of immobilization is greater with 5-nitroxide stearate and more marked in the case of bovine serum albumin

o-Phthaldehyde dramatically alters the association of both 5-nitroxide stearate and 16-nitroxide stearate with bovine serum albumin and ghosts In the case of bovine serum albumin, the ESR spectra indicate complete displacement of both spin probes into the buffer (Fig. 2) Erythrocytes behave almost identically upon *o*-phthaldehyde treatment Spectral analysis shows that about 95 % of both spin probes is displaced, but a small, significant proportion of label remains immobilized (Fig 1) Centrifugal separation of ghosts and buffer after *o*-phthaldehyde treatment shows that the immobilized spin probes remain membrane associated and that the free signal arises from probes displaced into the buffer (Fig 4)

What mechanisms participate in the probe displacement? In the case of bovine serum albumin, one can invoke only alterations of amino acid residues Since treatment of bovine serum albumin with sulphydryl blockers neither simulates nor modifies the *o*-phthaldehyde effect, one can eliminate participation of cysteine sulphydryls (through formation of thiohemiacetals and/or thioethers with *o*-phthaldehyde) Involvement of arginine residues appears more likely, particularly since aldehyde modification of arginines has been shown to interfere with the binding of 1-anilino-8-naphthalene sulfonate in the hydrophobic pocket of bovine serum albumin [15] Our observations that nitroxide stearates form complexes with arginine, that arginine reacts with *o*-phthaldehyde and that *o*-phthaldehyde disrupts the arginine–nitroxide stearate complexes support this argument. However, arginine modification cannot be the only mechanism since reaction of bovine serum albumin arginines with glyoxal, a smaller and less lipophilic dialdehyde than *o*-phthaldehyde, interferes with the binding of 1-anilino-8-naphthalene sulfonate but does not produce displacement of nitroxide stearates

The situation with ghosts is more complex, since nitroxide stearates might associate with both ghost proteins and lipids, since there are multiple ghost proteins, most of them reacting with *o*-phthaldehyde [20] and since our own data show that ghost phosphatidylethanolamine also reacts with *o*-phthaldehyde However, our experiments on liposomes prepared with the total lipids (including *o*-phthaldehyde–phosphatidylethanolamine) from *o*-phthaldehyde-treated ghosts, as well as on phosphatidylethanolamine-lecithin multibilayers reacted with *o*-phthaldehyde, clearly show that *o*-phthaldehyde modification does not effect displacement of nitroxide stearate from such lipid arrays These data also show that mere elimination of cationic ethanolamine groups is not sufficient for nitroxide stearate extrusion In this connection we

again stress that in ghosts, unlike bovine serum albumin, small amounts of spin probe remain bound after *o*-phthaldehyde treatment. With 16-nitroxide stearate, one can distinguish between probes associated with lipid bilayers and probes lying at a lipid–protein boundaries [4]; the residue spectrum resembles the latter case.

The lack of response with glyoxal, however, indicates that mere elimination of possible cationic sites whether lipid or protein, participating in the binding of the nitroxide stearates, cannot account for the effect of *o*-phthaldehyde Probe binding thus appears to include a large hydrophobic element.

Turning to the role of membrane proteins, we detect a significant difference in comparison to bovine serum albumin. PCMB, although it does not mimic the *o*-phthaldehyde effect by itself, reduces it by 60–70 % This suggests that *o*-phthaldehyde reaction with sulphydryl residues alters the architecture of membrane proteins in a way that reduces nitroxide stearate binding. Indeed, a large part of the *o*-phthaldehyde effect appears to involve sulphydryl modification This notion finds support in the ESR observation that MSL reports large changes by *o*-phthaldehyde of membrane protein sulphydryl and in laser–Raman experiments demonstrating reactions of thiols with *o*-phthaldehyde. Our data indicate that not all of the *o*-phthaldehyde induced displacement of nitroxide stearate involves sulphydryl modification and we suggest that mechanisms such as operate in bovine serum albumin are also involved

Since erythrocyte membranes comprise approx 40 % lipid by weight and liposomes produced from total membrane lipids readily accommodate nitroxide stearates, how can protein modifications account for the massive extrusion of nitroxide stearate from *o*-phthaldehyde treated erythrocytes ghosts? An explanation comes from our paramagnetic quenching studies.

Paramagnetic quenching of fluorescence by nitroxides occurs by transfer of excitation energy from the lowest singlet state of a fluorophore to the paramagnetic free radical [27]. This requires that two groups approach to within approx 6 Å [27, 28]. Our data (Fig. 10) clearly show that such contacts occur in both bovine serum albumin and erythrocyte ghosts and indicate that bovine serum albumin and certain ghost proteins contain binding sites where the nitroxide groups of 5-nitroxide stearate or 16-nitroxide stearate come into close apposition with tryptophans and/or tyrosines. (Although we observe tryptophan emission only, some of the paramagnetic quenching could be due to tyrosine deactivation This is because much of tryptophan excitation in both bovine serum albumin and ghosts derives from tyrosines by resonant energy transfer. Paramagnetic quenching of tyrosine would thus produce a decrease in tryptophan emission).

Bovine serum albumin and ghosts yield dissimilar quenching responses with increasing nitroxide/protein ratios in the reaction mixtures (Fig. 10) The quenching effect in bovine serum albumin appears saturable and the quenching plateau indicates a fixed number of binding sites per molecule The ghosts, in contrast, yield curves indicating two classes of binding. The first appears saturable at low concentrations; the second is non-saturable under our conditions

We suggest the following simple, but not exclusive interpretation. Quenching at low nitroxide concentrations derives largely from the presence of tryptophans and/or tyrosines at high-affinity (protein) binding sites and that non-saturable component represents partitioning of the spin label into a membrane lipid phase. Paramagnetic quenching in the latter case might occur via diffusion-limited contacts between

spin-label probes within lipid phases of restricted dimensions and proteins Perturbations of protein structure by the spin probes [29] cannot be excluded We will report more extensively on this matter (manuscript in preparation), but here emphasize that we did our ESR experiments under conditions favoring expression of possible, high-affinity binding sites.

We suspect, therefore, that the spin probes displaced by *o*-phthaldehyde came from apolar protein–lipid interfaces in erythrocyte ghosts and conclude that some of the membrane proteins of erythrocyte ghosts are surrounded by a boundary layer of membrane lipids Colley et al [30] have reasoned similarly from their observations on the effects of benzyl alcohol on erythrocyte membranes However our approach can yield more specific insights into the mechanisms involved in lipid protein interactions Furthermore, our data allow us to conclude that the state of membrane proteins can influence the lipids in the boundary layer and suggest an analogy between lipid–protein interactions in erythrocyte membranes and bovine serum albumin

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