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ELECTRON PARAMAGNETIC RESONANCE STUDIES OF MEMBRANE PROTEINS IN HEPATIC MICROSOMES

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Hepatic microsomal membranes, prepared under various conditions that yield either 'intact' or 'disrupted' microsomal vesicles, have been labeled via the sulfhydryl groups of intrinsic membrane proteins using nitroxide analogs of *N*-ethylmaleimide. Electron paramagnetic resonance spectra revealed the presence of two dominant classes of bound label corresponding to differing degrees of immobilization, the ratio of which were quantitated using a parameter designated the '*W*/*S*' ratio. For latent microsomes, the value of this parameter was determined to be 0.65 ± 0.02 and was influenced by factors such as label/protein ratio, incubation period, nitroxide structure, temperature and pH. The *W*/*S* ratio was also sensitive to the degree of membrane integrity as revealed by the latency of mannose 6-phosphate activity of glucose-6-phosphohydrolase. In addition, membrane disruption resulted in a corresponding decrease in the order parameter for nitroxide-labeled fatty acids intercalated within the lipid bilayer. The *W*/*S* ratio was observed to be dependent upon the method of microsome preparation yielding values of 1.02 ± 0.02 for 'hypertonically disrupted' vesicles and 1.28 ± 0.02 for 'mechanically disrupted' vesicles. Microsomal marker enzymes such as cytochrome *P*-450 and FAD-containing monooxygenase retained significant levels of functionality following nitroxide incorporation.

Introduction

Electron paramagnetic resonance (EPR) spectra of stable nitroxide-free radicals, incorporated both covalently and noncovalently into either synthetic or biological membranes, have been extensively applied to monitor alterations in bilayer fluidity or architecture [1].

Covalent modification of intrinsic membrane proteins by spin-label analogs provides a convenient method for investigating protein-protein and protein-lipid interactions and for monitoring changes in these interactions following membrane perturbation. These types of study have been successfully used with erythrocyte ghosts to define changes in membrane organization as a result of such phenomena as membrane damage by O_2^- [2], hemoglobin binding [3], the occurrence of pathological states involved in neurological disease [4] and cellular aging [5]. More recently, it has been demonstrated that the nature of the nitroxide probe and the conditions used to incorporate spin labels into erythrocyte ghost proteins can have a significant impact on the accuracy and reproducibility of these results [6].

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Abbreviations: Bicine, *N*,*N*-bis-(2-hydroxyethyl)glycine; CAPS, cyclohexylaminopropanesulfonic acid; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; Hepes, *N*-2-hydroxylethylpiperazine-*N*'-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid; Mal-6, 4-maleimide-2,2,6,6-tetramethylpiperidinooxyl.

The attempt to correlate the influence of membrane structure on the associated enzyme activities is complex and requires both suitable enzyme assays and an indicator of membrane integrity or organization. In contrast to the erythrocyte membrane, microsomal vesicles possess a number of well-defined membrane-bound enzymatic components such as the mixed-function oxidases. In this paper, we present details of the reproducible incorporation of nitroxide spin labels into microsomal vesicles and demonstrate these probes to be sensitive indicators of changes in protein organization and membrane integrity.

Materials and Methods

General. 4-Maleimido-2,2,6,6-tetramethylpiperidinooxyl (Mal-6), the maleimide-pyrrolidinyloxyl analogs shown in Fig. 3, 2,2,6,6-tetramethylpiperidinooxyl (TEMPO), 5-doxyl stearic acid and the corresponding methyl ester were obtained from Aldrich Chemical Company, Milwaukee, WI and were used without further purification. Previous work by Butterfield and Markesbery [7] have shown Mal-6 to be devoid of the isomaleimide isomer. *N*-Ethylmaleimide, iodoacetamide, *p*-hydroxymercuribenzoate, *p*-aminophenylmercuriacetate, Triton X-100, NADH, NADPH, sodium deoxycholate, mannose 6-phosphate, EDTA, Bicine, CAPS, CHES, Hepes and Mes were obtained from Sigma Chemical Company, St. Louis, MO. All other reagents were of the best commercially available grade.

Microsome preparation. All procedures were performed at 0–4 °C. Microsomes were isolated from the livers of male Sprague-Dawley rats (125–300 g) and male Fischer F-344 rats (125–200 g) maintained on a standard diet (Purina Rodent Chow No. 5001). Rats were killed by decapitation, the livers were rapidly excised and placed in 0.25 M sucrose. Latent * microsomal vesicles were prepared essentially as described by Phillips and Ness [8]. This procedure involves mild tissue homogenization and avoids lysozome rupture and concomitant proteolysis. Liver samples (1 g/10 ml 0.25 M sucrose) were homogenized by four or five passes of a Dounce homogenizer (small pestle) and centrifuged at $11\,000 \times g$ for 15 min. The supernatant was decanted, centrifuged a second time under identical conditions and the upper 65% of the supernatant was retained. Microsomal vesicles were pelleted at $100\,000 \times g$ for 45 min, washed with 0.25 M sucrose and resuspended at a protein concentration of 20–25 mg/ml. Microsomes prepared using this protocol, referred to as intact microsomes have been shown to be free of contaminating lysozomes, as indicated by acid phosphatase assays. Modified or disrupted microsomal vesicles, exhibiting a markedly decreased level of mannose-6-phosphatase latency, were prepared using alternative procedures. In these cases, not only is the degree of tissue disruption increased but the failure to completely exclude lysozome contamination may result in proteolysis. Liver samples (1 g/4 ml buffer) were homogenized in a phosphate/sucrose/EDTA/KCl medium, as described by Heller and Gould [9] and were termed hypertonically disrupted vesicles. Mechanically disrupted microsomes were prepared by homogenization of tissue (1 g/3 ml 0.25 M sucrose) using a model PT polytron (speed 5 for 3 s, Brinkman Instruments Company, Westburg, NY). The homogenate was then spun at $10\,000 \times g$ for 15 min and the supernatant was retained. Microsomal vesicles were then pelleted by centrifugation at $100\,000 \times g$ for 40 min, washed with 0.25 M sucrose and resuspended at a protein concentration of 15–20 mg/ml. The integrity of the microsomal preparations were determined using the latency of mannose-6-phosphate phosphohydrolase activity of glucose 6-phosphate [10]. Resolution of the intact microsomal vesicles into integral and peripheral protein components was achieved using a modification of the method of Fujiki et al. [11]. Microsomes were diluted to a final protein concentration of 1 mg/ml with 100 mM sodium carbonate (pH 11.5) and incubated at 0 °C for 30 min. The microsomal suspension was acidified to pH 6.5 with 1 M acetic acid and centrifuged at $100\,000 \times g$ for 45 min. The supernatant was de-

* Latency: In this paper, latency is designed to convey a quantitative measure of the integrity of the microsomal membrane. The stereoselectivity of the glucose 6-phosphate transport system ensures only mannose 6-phosphate that is able to diffuse into the interior of the vesicle due to a permeant membrane will be subject to hydrolysis. Thus, latency is the ratio of the concentration of inorganic phosphate produced by detergent-disrupted vesicles to that produced in the absence of detergent.

canted, concentrated by pressure filtration and dialyzed against 0.25 M sucrose. The disrupted vesicles, indicated by the absence of mannose 6-phosphate latency, were washed and resuspended in 0.25 M sucrose. Protein was assayed by a Biuret method [12] using bovine serum albumin as standard.

Preparation of nitroxide-modified microsomes. Covalent modification of membrane proteins by nitroxide-maleimide analogs was achieved by incubating the microsomal vesicles with Mal-6 (1 mg Mal-6/25 mg protein) or alternative spin-labeled derivatives in the same molar/protein ratio, at 4°C for 16 h in the absence of light, following which, excess unreacted spin label was removed by centrifugation and the microsomes were washed twice and resuspended in sucrose at a protein concentration of 20–25 mg/ml.

Incorporation of 5-doxyl stearic acid or the corresponding methyl ester was achieved by evaporating a thin film of the fatty acid in ethanol on the inner surface of a glass vial using a stream of purified nitrogen gas, following which, the microsomal suspension was added and gently vortexed for 5 min at 4°C. Fatty acid spin labels were added in the ratio 1 μg spin label/mg protein. This ratio has been shown by Sauerheber et al. [13] to result in optimum incorporation of fatty acid spin labels. In addition, m values calculated for the 5-doxyl stearic acid label in intact microsomes were consistently greater than 0.96, indicative of negligible probe-probe interactions.

EPR spectroscopy. EPR spectra were recorded using either a Varian E9 or E109 spectrometer (Varian Associates, Palo Alto, CA) operating at 9 GHz and equipped with a variable temperature accessory. Microsomal samples were routinely examined, using either 1 mm diameter quartz EPR tubes (Wilmad Glass Company, Bueno, NJ) or a standard flat cell, employing 14 mW microwave power and a modulation amplitude of 0.063 mT. Samples were prepared in duplicate and the average of three scans was used for analysis.

Order parameters were calculated from the spectra for 5-doxyl stearic acid using the equation [14]:

$$S = \frac{T_{\parallel} - (T_{\perp} + 0.86)}{T_{zz} - 0.5(T_{xx} + T_{yy})}$$

where $T_{\parallel}$ and $T_{\perp}$ are related to the maximum and minimum hyperfine splittings measured from the experimental spectra (see Fig. 7). $T_{xx} = 6.3$ G, $T_{yy} = 5.8$ G and $T_{zz} = 33.6$ G [15]. The order parameter provides a measure of the mean angular deviation of the hydrocarbon chains from their time-averaged orientation in the lipid bilayer. High values of S reflect a highly ordered structure.

Biochemical studies. Determination of FAD-containing monooxygenase activity was achieved as methimazole-*S*-oxidase activity by following NADPH oxidation as described by Cavagnaro et al. [16]. Cytochrome *P*-450 content was assessed by the method of Omura and Sato [17]. Aniline hydroxylase activity was determined according to the method of O'Brien and Rahimtula [18]. *p*-Nitroanisole-*O*-demethylation and aminopyrine-*N*-demethylation activities were assayed as described in the literature [19].

Results

Reaction of the spin label, Mal-6, with intact (greater than 90% latent) hepatic microsomes prepared using 0.25 M sucrose yielded the EPR spectrum shown in Fig. 1. This type of spectrum is very similar to that obtained for other membrane systems, such as erythrocyte ghosts, following incorporation of Mal-6 and shows the presence of two dominant classes of binding site. One exhibiting a high degree of immobilization, represented by the broad low-field peak labeled '*S*' and a second, less immobilized class, characterized by the relatively narrow feature labeled '*W*' *. Previous work has demonstrated the ratio of the populations of these two classes of bound label, typified by the relative peak heights and referred to as the *W/S* ratio, to be an extremely sensitive indicator of changes in protein environment and thus membrane organization [6]. Analysis of the EPR spectrum obtained for intact microsomes yielded a

* In previous studies using Mal-6, the '*W/S*' ratio has referred to the ratio of the signal amplitudes of the low-field features of both the weakly and strongly immobilized classes of spin label. However, owing to the incomplete resolution of the low-field feature of the weakly immobilized class of nitroxides in intact microsomes (compare Fig. 1B and C), we have utilized the amplitude of the high-field peak, exhibiting increased resolution.

W/S ratio of 0.65 ± 0.02 at 19°C in 0.25 M sucrose. Control samples of microsomal vesicles subjected to identical incubation and washing steps, in the absence of Mal-6, retained greater than 85% latency, whereas Mal-6-labeled microsomes exhibited greater than 70% retention.

Prior incubation of intact microsomal membranes with non-spin-labeled reagents directed against free sulfhydryl groups followed by incubation with Mal-6 resulted in changes in the observed EPR spectra. Modification of the vesicles with *p*-hydroxymercuribenzoate (500 μM) for 1 h at 0°C resulted in no apparent binding of Mal-6, as evident from the absence of any detectable EPR signal. Identical results were obtained if the microsomes were initially treated with *p*-aminophenylmercuriacetate. In contrast, if the membranes were preincubated with iodoacetamide (500 μM), the resulting EPR spectrum was similar in lineshape to those of the control Mal-6 membranes, but with a decreased W/S ratio of 0.39. Prior treatment of the membranes with *N*-ethylmaleimide (500 μM) followed by reaction with Mal-6 yielded a spectrum with a W/S ratio of 0.27 with only a minor component due to the weakly immobilized class of labels. Several experimental parameters were observed to have a profound influence on the EPR spectra of the labeled membranes. Increasing the reaction time between the spin label and the microsomes at a constant probe/protein ratio (1 mg Mal-6/25 mg protein) resulted in a progressive decrease in the W/S ratio as shown in Fig. 2A. The W/S ratio was observed to decrease from 1.33 following a 1-h incubation to a limiting value

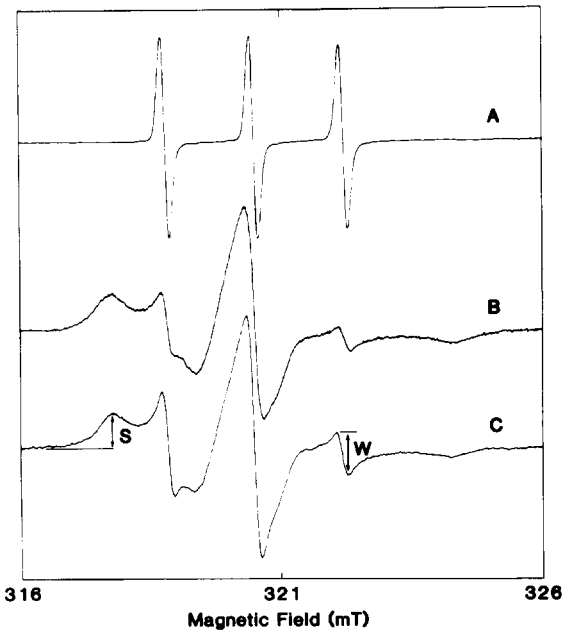


Fig. 1. EPR spectra of free and membrane-bound Mal-6. (A) Free spin label (0.1 mM) in 0.25 M sucrose. (B) Spin-labeled intact microsomal vesicles (10 mg/ml) in 0.25 M sucrose. (C) Spin-labeled mechanically disrupted microsomal vesicles (15 mg/ml) in 0.25 M sucrose. Spectra were recorded at 19°C using 14 mW microwave power and a modulation amplitude of 0.063 mT.

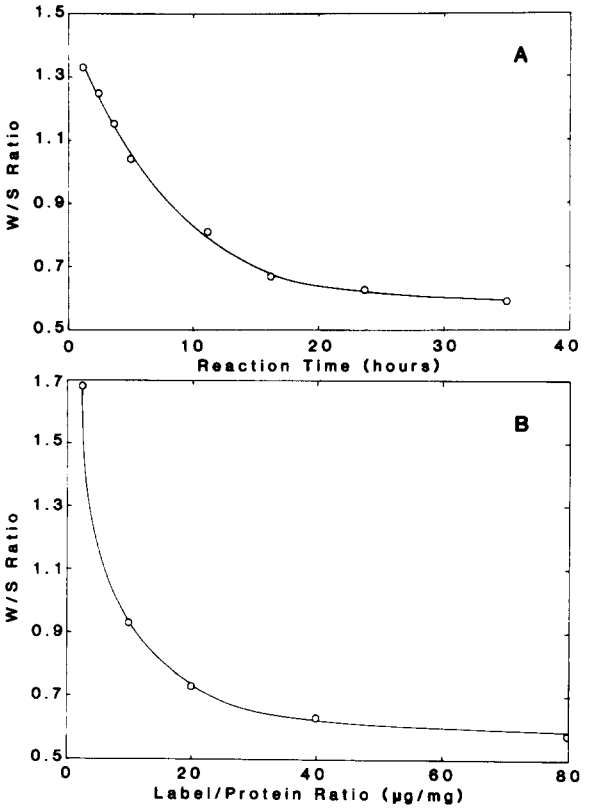


Fig. 2. Influence of incubation time and label/protein stoichiometry on the W/S ratio. (A) Intact microsomal vesicles were incubated with Mal-6 at a ratio of 1 mg/25 mg protein in 0.25 M sucrose at 4°C for varying time intervals. The microsomes were extensively washed with sucrose and the W/S ratio was determined from the experimental EPR spectra recorded under conditions described in Fig. 1. (B) Intact microsomal vesicles were incubated for 16 h at 4°C with Mal-6 at varying label/protein ratios in 0.25 M sucrose, extensively washed and the W/S ratio was determined from the experimental EPR spectra.

of approx. 0.59 at 35 h. A similar pattern was observed under conditions of constant reaction time (16 h) and a variable label/protein ratio (Fig. 2B). Maximum and minimum *W/S* values of 1.69 and 0.57 were obtained at Mal-6/protein ratios of 2 and 80 μg/mg, respectively.

The structure of the nitroxide spin label, both in terms of the number of carbon atoms in the nitroxide ring and the number of atoms separating the nitroxide and maleimide functions was also found to perturb the *W/S* ratio. Changing the nitroxide from a six-membered piperidinooxyl structure to a five-membered pyrrolidinyloxyl derivative resulted in an increase in the *W/S* ratio from 0.65 to 0.72. This ratio was also increased following inclusion of additional 'spacer' elements between the two functional domains of the spin label as shown in Figs. 3 and 4.

To attempt to define the location of the Mal-6-labeled proteins, we utilized carbonate treatment of the intact labeled microsomes to separate the protein constituents into integral and peripheral components. The EPR spectra of the resuspended carbonate-treated vesicles and the resolved peripheral proteins are compared in Fig. 5. Double integration of the EPR signal intensities indicated a label/protein ratio approx. 10-times greater for the integral proteins compared to that for the peripheral proteins.

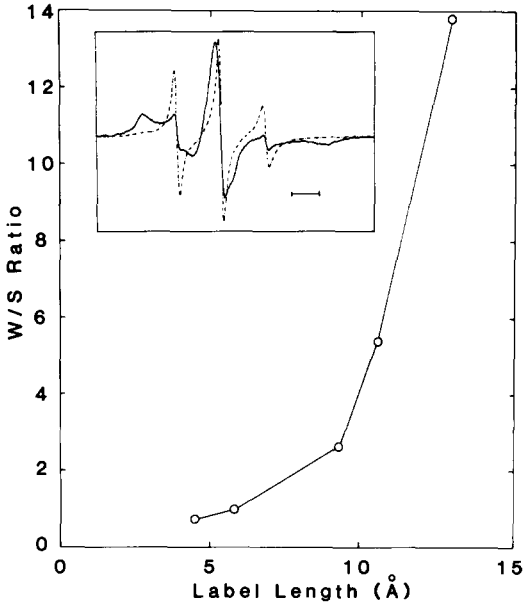


Fig. 4. Effect of increasing chain-length on the *W/S* ratio of pyrrolidinyloxyl maleimide spin labels incorporated into intact microsomal vesicles. The inset shows the EPR spectra obtained from microsomes labeled with the shortest (———) and longest (·–·–·) chain derivatives. The field marker represents 1 mT.

Addition of ascorbate (final concentration 2

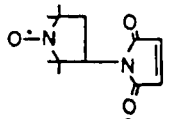
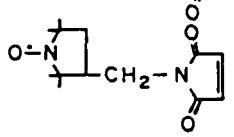
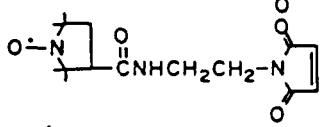
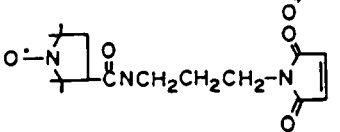
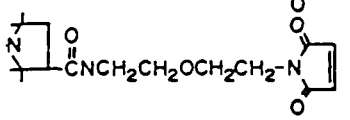
Structure	Systematic Name	Length Å	W/S Ratio
	3-Maleimido-3,3,5,5-tetramethyl-1-pyrrolidinyloxyl	4.4	0.70
	3-(Maleimidomethyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl	5.7	0.92
	3-(2-(Maleimidoethyl)carbamoyl-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl	9.3	2.64
	3-(2-(2-Maleimidopropyl)-carbamoyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl	10.5	5.44
	3-(2-(2-(Maleimidoethoxy)-ethyl)carbamyl)-2,2,5,5-tetramethyl-1-pyrrolidinyloxyl	12.9	13.87

Fig. 3. Chemical structures of pyrrolidinyloxyl maleimide spin labels. Intact microsomes were spin-labeled and the EPR spectra were obtained as described in Materials and Methods. Label dimensions were taken from Delmelle and Virmaux [21].

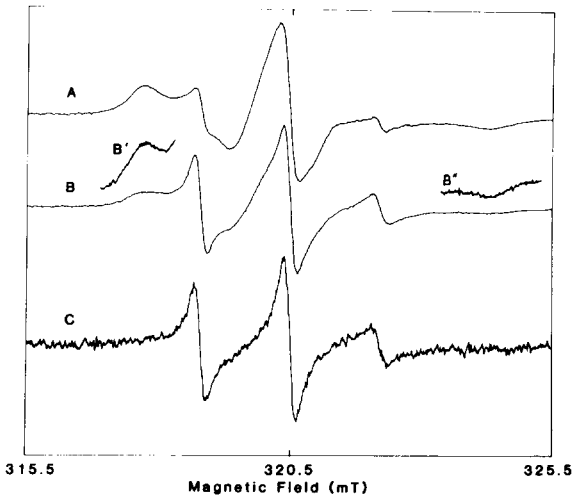


Fig. 5. EPR spectra of Mal-6-labeled integral and peripheral microsomal proteins. (A) Mal-6-labeled intact vesicles (32 mg protein/ml) in 0.25 M sucrose. (B) Integral labeled proteins present in carbonate-treated vesicles (21 mg protein/ml) resuspended in 0.25 M sucrose. The insets, B′ and B″ represent the low- and high-field features of the spectrum recorded at higher gain. (C) Peripheral labeled proteins isolated from carbonate-treated vesicles (14 mg protein/ml) in 0.25 M sucrose.

mM) to Mal-6-labeled intact microsomes resulted in complete abolition of the EPR spectrum, indicating reduction of the nitroxide to the corresponding hydroxylamine. Similar results were observed following addition of dithionite. However, incubation of the microsomes with either NADH or NADPH (final concentration 1 mM) appeared to have no effect.

Storage of labeled intact microsomes at 4°C showed no deterioration in the EPR spectrum up to 40 h. However, longer intervals resulted in a gradual decrease in the W/S ratio, yielding a value of 0.55 at 64 h with apparent microsome agregation. Labeled membranes subjected to a simple cycle of freezing at 77 K followed by rapid thawing to room temperature showed only a very slight elevation of the W/S ratio to 0.67. Mannose-6-phosphatase activities of unlabeled microsomes subjected to an identical cycle indicated the retention of greater than 90% latency.

A considerable number of procedures have been published for the preparation of microsomal vesicles, the majority of which utilize different methods of tissue homogenization and alternate media. EPR spectra were obtained for Mal-6-labeled microsomes isolated by two different methods that result in partially disrupted vesicles and the W/S ratios compared with those of the intact form. The W/S ratio was significantly increased both for hypertonically disrupted and mechanically disrupted vesicles, yielding values of 1.05 ± 0.02 and 1.28 ± 0.02, respectively. In addition, the latency of mannose-6-phosphatase indicated the retention of approx. 52 and 34% of the integrity of control values. Hypertonic-treatment of intact-labeled vesicles resulted in an increase in

TABLE I

ENZYMATIC ACTIVITIES OF SPIN-LABELED HEPATIC MICROSOMES

Enzyme activity [a]	Microsomes ($n = 5$)	Mal-6-microsomes [c] ($n = 5$)	Change [d] (from control)
FAD-containing monooxygenase	2.11 ± 0.18	1.49 ± 0.18	−29%
Cytochrome *P*-450 content [b]	0.57 ± 0.05	0.49 ± 0.04	−15%
Aniline hydroxylase	5.69 ± 1.20	5.33 ± 0.45	n.s.
p-Nitroanisole-*O*-demethylase	0.78 ± 0.15	0.67 ± 0.14	n.s.
Aminopyrine-*N*-demethylase	4.65 ± 0.25	3.98 ± 0.40	−14%

[a] Enzyme activities are given in nmol/min per mg protein.
[b] Cytochrome *P*-450 content is given in nmol/mg protein.
[c] Hepatic microsomes labeled with maleimide spin label as described in Materials and Methods.
[d] Significance is where $P < 0.05$. n.s., not significant.

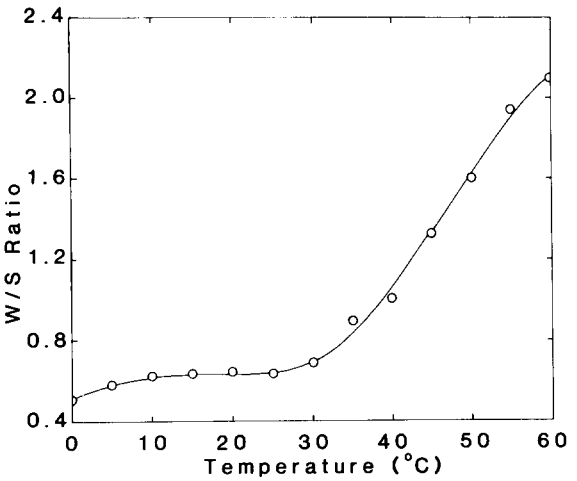


Fig. 6. Temperature dependence of the *W/S* ratio of Mal-6 incorporated into intact microsomal vesicles. Microsomes in 0.25 M sucrose were incubated with Mal-6 (1 mg/25 mg protein) for 16 h at 4 °C, extensively washed with sucrose and the EPR spetrum was recorded as described in Materials and Methods. Membrane samples were maintained at the indicated temperatures for 10 min prior to spectra aquisition.

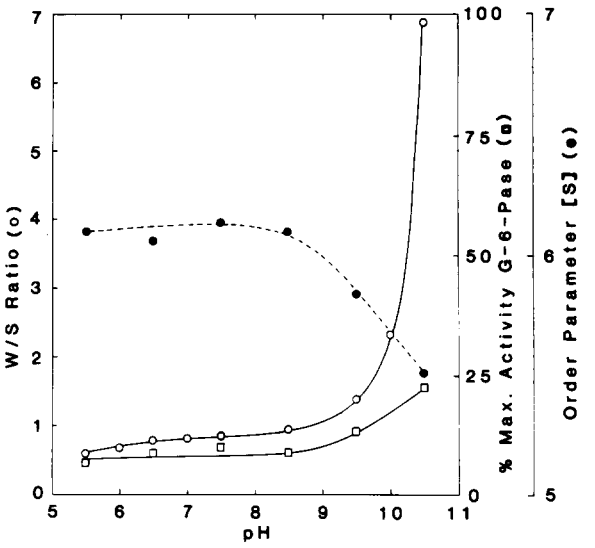


Fig. 7. pH Dependence of the *W/S* ratio of Mal-6-labeled intact microsomal vesicles. Labeled vesicles (10 mg protein/ml) were suspended in 0.25 M sucrose containing various zwitterionic buffers (final concentration 0.25 M). Buffers used and their respective pH values were: Mes, pH 5.5–7.0; Hepes, pH 7.5; Bicine, pH 8.5; CHES, pH 9.5–10.0; CAPS, pH 10.5.

the *W/S* ratio to 0.78. However, in contrast, mechanical treatment resulted in no significant change.

The activities of representative microsomal marker enzymes for intact microsomes prior to, and following Mal-6 incorporation, are shown in Table I. Both cytochrome *P*-450 content and aminopyrine-*N*-demethylase activity were depressed by approx. 15% of control, following nitroxide modification, whereas values for FAD-containing monooxygenase indicated a 29% decrease in activity. In contrast, aniline hydroxylase and *p*-nitroanisole-*O*-demethylase activities remained unchanged.

The influence of temperature on the nitroxide label mobility is shown in Fig. 6. The *W/S* ratio was relatively constant within the range 10–25 °C. However, above 30 °C, the *W/S* ratio rapidly increased, reflecting both a change in the structure of the lipid bilayer and the possible onset of thermally induced protein denaturation. Increased temperature also resulted in a decrease in the separation of the two outer hyperfine lines from 6.60 mT at 20 °C to 6.39 mT and 60 °C.

The pH dependence of the *W/S* ratio for Mal-6-labeled intact microsomes is shown in Fig. 7. Whilst relatively contant at values below pH 7.5, more alkaline conditions caused a significant, irreversible, increase in the ratio. To examine whether these changes in the *W/S* ratio reflected alterations in membrane-lipid fluidity, changes in the membrane integrity or pH-induced denaturation of selected proteins, both lipid fluidity and vesicle integrity were examined using lipid-soluble nitroxide probes and the latency of mannose-6-phosphatase activity, respectively. Attempts to monitor changes in membrane fluidity via the partitioning of the spin label TEMPO between the aqueous and hydrocarbon phases proved unsuccessful. Addition of TEMPO to intact microsomes resulted in a spectrum directly comparable to that of the spin label in an aqueous environment, indicating very little partitioning of the probe into the lipid bilayer. In contrast, addition of the nitroxide fatty acid derivative, 5-doxyl stearic acid or its corresponding methyl ester, resulted in near complete incorporation into the membrane, as shown in Fig. 8. Exposure of the intact microsomes to progressively alkaline conditions resulted in a decrease in the order parameter (*S*). Similarly, above pH 8.5, there was a progressive decrease in the degree of

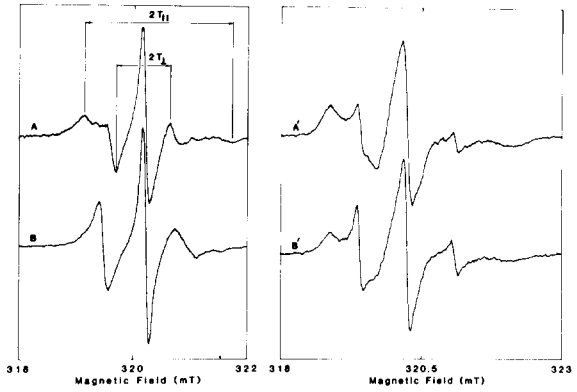


Fig. 8. Effect of membrane disruption on the EPR spectra of 5-doxyl stearic acid and Mal-6 incorporated into intact microsomal vesicles. (A) Microsomal membranes (29 mg protein/ml) were incubated with 5-doxyl stearic acid in the ratio 1.1 μg fatty acid/mg protein in 0.25 M sucrose and the EPR spectrum was recorded as described in Materials and Methods. (B) Spectrum obtained following addition of deoxycholate (final concentration 2%, w/v). (A′) Mal-6-labeled microsomes (10 mg protein/ml) in 0.25 M sucrose. (B′) Spectrum obtained following addition of deoxycholate (final concentration 2%, w/v).

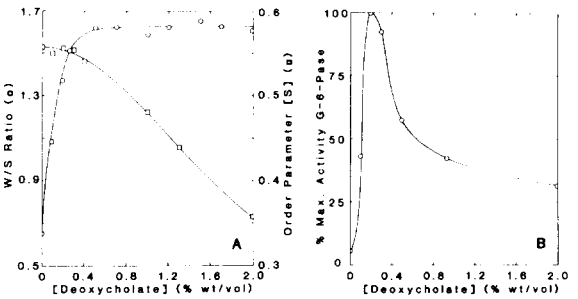


Fig. 9. Influence of membrane disruption on the spectroscopic properties of Mal-6 and 5-doxyl stearic acid spin labels incorporated into intact microsomal vesicles and the latency of glucose-6-phosphatase activity. *W/S* ratios, the order parameter (*S*) and the mannose-6-phosphate phosphohydrolase activity of glucose-6-phosphatase were determined as described in the text.

microsome latency. However, at pH 10.5, the vesicles still retained approx. 78% of their original integrity.

To compare the effects of microsomal membrane integrity on the protein and lipid components, Mal-6-labeled vesicles and vesicles incorporating 5-doxyl stearic acid were disrupted by detergent addition, vesicle integrity being monitored by mannose-6-phosphatase latency. Representative EPR spectra comparing intact and detergent-disrupted microsomes are shown in Fig. 8, while the corresponding variations in the *W/S* ratio, order parameter and relative mannose-6-phosphatase activity are shown in Fig. 9. Low levels of detergent were observed to produce a dramatic increase in the *W/S* ratio, reaching a limiting value of approx. 1.64 at 0.5% deoxycholate and remaining constant at higher detergent concentrations (Fig. 9A). Alterations in membrane fluidity were also reflected in a corresponding decrease in the order parameter. However, the order parameter was not as responsive to the initial phases of membrane disruption and did not attain a limiting value within the deoxycholate concentrations examined. Vesicle disruption as indicated by the decrease in mannose-6-phosphatase latency is shown in Fig. 9B. Low detergent levels caused a rapid destruction of the membrane integrity. However, the inhibition of mannose-6-phosphatase [20], evident at higher detergent concentrations, resulted in an apparent partial restoration of vesicle integrity.

Discussion

The preceding results demonstrate a reliable and reproducible technique for the covalent incorporation of nitroxide spin labels into microsomal vesicles. While we have been unable to identify the individual labeled polypeptides, we have attempted to define some of the characteristics of the labeled proteins and identify conditions that perturb the observed EPR spectra. The prior reaction of microsomal membranes with Hg derivatives to effect modification of accessible sulfhydryl groups (cysteine) followed by reaction with spin label resulted in the absence of any detectable Mal-6 incorporation, indicating the nitroxide derivative did not react with alternate protein sidechains, such as the amino groups of lysine residues, to any significant extent. In contrast, incubation of the membranes with either *N*-ethylmaleimide or iodoacetamide did not preclude subsequent Mal-6 incorporation under the conditions used, although changes in the resultant EPR spectra suggested that the nonparamagnetic compounds initially reacted with the more mobile sulfhydryl components. The results of the treat-

ment of Mal-6-labeled intact vesicles with carbonate demonstrated that both integral and peripheral membrane proteins were modified with a high proportion of the labeled sites occurring on integral proteins. Note, Fujiki et al. [12] have demonstrated that approx. 53% of the total protein (integral and peripheral) remains associated with the lipid bilayer following carbonate treatment. The resuspended, membrane-bound integral protein fraction retained both strongly and weakly immobilized nitroxide labels. However, in contrast, the peripheral proteins exhibited only the weakly immobilized component.

Exposure of the labeled intact vesicles to ascorbate effected total reduction of the nitroxides to their corresponding hydroxylamines, indicating the modified protein residues to be readily accessible to solvent and thus presumably located on the cytoplasmic surface of the membrane. Increasing the separation between the nitroxide and the maleimide functions had a considerable influence on the resultant EPR spectrum. Chain-lengths greater than approx. 6 Å produced significantly higher W/S ratios, suggesting that some of the sulfhydryl groups could be situated either within protein clefts or on protein residues embedded within the membrane.

The W/S ratio of the labeled microsomes was found to be highly dependent upon the conditions used to achieve Mal-6 incorporation. Of particular consideration were the results of the incubation time and label/protein ratio experiments. These data indicate a degree of differential reactivity of the accessible sulfhydryl groups, with the strongly immobilized sites exhibiting lower reactivity. Mal-6 modification of both integral and peripheral membrane proteins had little effect on the integrity of the membrane structure, as judged by the retention of mannose-6-phosphatase latency. However, the methods used to isolate the microsomal vesicles had a significant influence both on the integrity of the membrane and the observed EPR spectra. Decreased membrane integrity was found to result in elevated W/S ratios. The absence of any significant effect on the W/S ratio of intact microsomes subjected to mechanical treatment suggests that the major changes in the W/S ratio and mannose-6-phosphatase latencies occurring during the preparation of vesicles using this technique are probably due to proteolytic disruption of the membrane which may expose additional sites for Mal-6 reaction, including any located on the luminal surface. In contrast, 'hypertonic' treatment of intact nitroxide-labeled microsomes increased the W/S ratio. This presumably results from a combination of the salt and chelation effects of the 'hypertonic' medium, resulting in a partial dissociation of both labeled and unlabeled peripheral membrane proteins.

Our results indicate that vesicle integrity could be maintained for a limited period of time at 4°C, whereas microsomes were stable indefinitely if rapidly frozen and stored under liquid nitrogen. Similar results have been reported by Nordlie et al. [22].

The elevation of the W/S ratio with increasing pH indicated a significant, irreversible perturbation of vesicle organization, commencing at approx. pH 8.5. Previous work has demonstrated the disruption of microsome integrity at alkaline pH [23]. However, although we observed a decrease in the latency of mannose-6-phosphatase above pH 9, the membranes retained a high degree of integrity up to and including pH 10.5. Total abolition of mannose-6-phosphatase latency could be achieved by incubating labeled microsomes in Na_2CO_3 at pH 11.5. In addition, the small decrease in the order parameter for the 5-doxyl stearic acid probe intercalated within intact microsomes, compared with the magnitude of the changes observed during detergent disruption, indicates that the change in the W/S ratio with increasing pH may predominantly reflect an alteration in protein structure due to pH-induced denaturation.

Incorporation of Mal-6 into intact vesicles resulted in decreased membrane-associated enzyme activity. Both cytochrome *P*-450 and FAD-containing monooxygenase incorporate thiol groups near the catalytic site [24,25] and in the case of the former, this enzyme has been localized on both the cytoplasmic and luminal surfaces [26]. The small decrease in cytochrome *P*-450 content and aminopyrine-demethylase activity coupled with the unchanged aniline hydroxylase and *p*-nitroanisole-*o*-demethylase activities indicate only a minor alteration in the structure of cytochrome *P*-450 following labelling. However, the markedly increased inhibition of FAD-containing monooxygenase ac-

tivity may result from modification of essential sulfhydryl groups located in close proximity to the active site.

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