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STRUCTURAL CHANGES OF RAT LIVER MICROSOMAL MEMBRANES INDUCED BY THE ORAL ADMINISTRATION OF CARBON TETRACHLORIDE

^{31}P-NMR AND SPIN-LABEL STUDIES*

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Abstract—The acute effects of carbon tetrachloride (CCl_4) on the membrane structure of rat liver microsomes were studied using ^{31}P-NMR and spin-labeling techniques. ^{31}P-NMR spectra of rat liver microsomes were not changed appreciably after the oral administration of CCl_4, indicating that the surface structures of microsomal membranes probably are not influenced by the oral administration of CCl_4. Four different spin-labeled stearic acids, 5-(*N*-oxyl-4′,4′-dimethyloxazolidine)-stearic acid (5SLS), its methyl ester (5SLSM), 12-(*N*-oxyl-4′,4′-dimethyloxazolidine)-stearic acid (12SLS) and its methyl ester (12SLSM), were used for the estimation of membrane fluidity. The apparent rotational correlation time of 12SLS decreased from 4.0 nsec to 3.0 nsec after the oral administration of CCl_4, while the order parameter of 5SLS did not change. The results suggest that CCl_4 or its metabolites increase the membrane fluidity of liver microsomes primarily at hydrophobic regions rather than at the surface layer. The ESR spectrum of 5SLSM in microsomal membranes comprised two different signals; one was an anisotropic signal and the other was a rather isotropic one. The ratio of the anisotropic signal to the isotropic one decreased markedly after the oral administration of CCl_4 and depended on the dose of CCl_4. The suitability of this ESR technique with 5SLSM for the estimation of membrane damage is discussed.

The oral administration of carbon tetrachloride (CCl_4) has a potent toxic effect on rat liver microsomes [1, 2]. The hepatic toxicity of CCl_4 has been investigated for many years, but the mechanism of liver injury and subsequent lethality is still unknown. CCl_4 is thought to be converted to the trichloromethyl radical (CCl_3) by the microsomal system [3]. The trichloromethyl radical produced may initiate lipid peroxidation by the abstraction of the methylene hydrogen from polyunsaturated fatty acids. The mechanism of the toxic action of lipid peroxidation seems to be by damage to microsomal membranes through cross-linking among membrane components [4, 5]. However, little is known about the structural changes in microsomal membranes which occur after CCl_4 administration.

Nuclear magnetic resonance (NMR) and spin-label (electron spin resonance) are widely used for the investigation of membrane structure and fluidity. ^{31}P-NMR provides information on the structural organization of the membrane lipids [6]. For instance, erythrocyte membranes show a bilayer type of ^{31}P-NMR spectrum for virtually all the phospholipids. However, it was demonstrated recently that the ^{31}P-NMR spectra of rabbit liver microsomes [7], of beef and rat liver endoplasmic reticulum [8], and of rat liver slices [9] are characteristic non-bilayer type, suggesting that a fraction of the membrane phospholipids undergoes an isotropic motion. Spin-labeling is a very sensitive technique [10], and many kinds of spin-probes have been developed for pharmacological studies [11]. Fatty acid derivatives with a nitroxide radical at various positions are used for the evaluation of membrane fluidity. Using 5-(*N*-oxyl-4′,4′-dimethyloxazolidine)-stearic acid; Stier and Sackmann [12] also suggested the presence of a phospholipid halo around the membrane protein in liver microsomes.

In this report we investigated the acute effect of CCl_4 on the membrane structure of rat liver microsomes using ^{31}P-NMR and spin-label. We found that CCl_4 or its metabolites caused a structural change in the microsomal membranes, particularly in the hydrophobic region of the membrane. During this investigation, we discovered that the methyl ester of spin-labeled fatty acid is a very suitable spin-probe for the estimation of membrane damage induced by CCl_4.

MATERIALS AND METHODS

Chemicals. Spin-probes, 5-(*N*-oxyl-4′,4′-dimethyloxazolidine)-stearic acid (5SLS) and its methyl ester (5SLSM), were synthesized by the method of Jost *et al.* [13]. 12-(*N*-Oxyl-4′,4′-dimethyloxazolidine)-stearic acid (12SLS) and its methyl ester (12SLSM) were synthesized by the method of Waggoner *et al.* [14]. Total lipids of the microsomes

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were prepared as described by Bligh and Dyer [15] and stored as a chloroform solution at −20°. All other chemicals were from commercial sources.

Preparation of microsomes. Male Wistar rats (100–200 g, Nihon Seibutsuzairyo Co., Tokyo, Japan) were used throughout as liver donors. Two milliliters of 1% carboxymethylcellulose sodium (CMC-Na) suspension containing carbon tetrachloride was administered orally at a dose of 0.15 to 2 ml CCl_4/kg body wt. Control rats were given 2 ml of 1% CMC-Na solution. After 3–30 hr, the rats were killed by cardiac puncture, and the liver was perfused with saline. Microsomes were prepared as described by Ariyoshi *et al.* [16] with a slight modification. The liver was homogenized with a Teflon–glass homogenizer in 3 vol. of 1.15% KCl per liver weight. The homogenate was centrifuged at 9000 *g* for 15 min, and the microsomes were separated from the resulting supernatant solution by centrifugation at 100,000 *g* for 60 min. The pellets obtained were resuspended with homogenizer in 2 ml of Tris–HCl-buffered 20% D_2O saline (pH 7.4) for NMR measurements, and in 2 ml of phosphate-buffered saline (pH 7.4) for ESR measurements. The concentration of protein in the microsomes was estimated by the method of Lowry *et al.* [17]. All preparations were kept in ice at each step.

Reconstitution of microsomal membranes. Reconstitution was performed by removal of Triton X-100 with Bio-Beads SM-2 (Bio-Rad Co.) as described by Gerritsen *et al.* [18]. Microsomes were solubilized with 1% Triton X-100 and mixed with different amounts of the microsomal total lipid suspension which was prepared previously by the vortexing method [19]. The mixture was incubated with Bio-Beads SM-2 at 4° for 24 hr to remove the detergent. The reconstituted vesicles prepared were used for ESR measurement.

^{31}P-NMR measurements. The microsomal suspension (26.6 mg protein in 2 ml of Tris–HCl-buffered 20% D_2O saline) was transferred under argon atmosphere into a sample tube (10 mm o.d.) sealed with a Teflon stopper and measured with ^{31}P-NMR spectroscopy. Proton-decoupled ^{31}P-NMR spectra were obtained at 40.25 MHz with a JEOL JNM-FX 100 spectrometer in the Fourier transform mode. The wide-band proton decoupling power was 5 W. An internal deuterium field frequency lock was used. The free induction decay signals were obtained from up to 10,000 transients, employing a 16 μsec r.f. pulse and a 1.5 sec interpulse time. The temperature was controlled at 37 ± 1°.

ESR measurements. Spin-labeled microsomes were prepared as follows. An aliquot of a spin-probe in methanol stored at −20° was transferred into a microtube and then the spin-probe was coated as a thin film by evaporating the solvent. Fifty microliters of microsomal suspension (13.3 mg protein/ml) was added to the microtube and vortexed for 1 min at room temperature. The ratio of a spin-probe to microsomal total lipids was approximately 1:200. This concentration of the spin-probe should not cause any significant line-broadening in the ESR spectrum [20]. The spin-labeled microsomes obtained were transferred into a disposable micropipette (100 μl), sealed at one end, and immediately measured with ESR spectroscopy. ESR spectra were recorded at 25 ± 1° on a JEOL PX-1 X-band spectrometer equipped with a temperature controller, JEOL JES-UCT-2AX. The amplitude of 100 (k) Hz field modulation was 2.0 G. The microwave power was kept at 4.0 mW. The magnetic field was 3260 ± 50 G and the hyperfine splitting value was calibrated with Mn^{2+}. Order parameter and apparent rotational correlation time were estimated from the ESR spectra as described by us [21] and by Keith *et al.* [22] respectively.

RESULTS

^{31}P-NMR studies of membrane structure. Liver microsomes from control or CCl_4-treated rats were

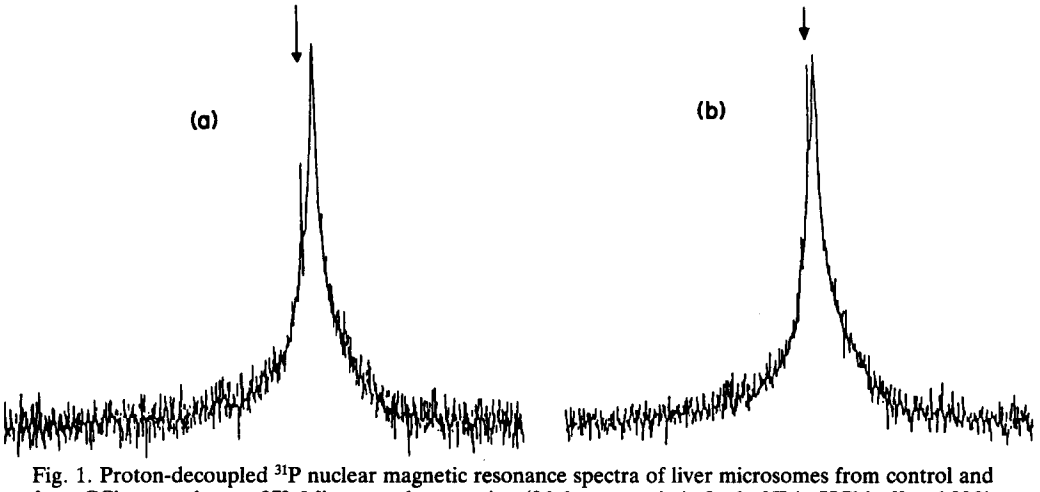

Fig. 1. Proton-decoupled ^{31}P nuclear magnetic resonance spectra of liver microsomes from control and from CCl_4-treated rat at 37°. Microsomal suspension (26.6 mg protein in 2 ml of Tris–HCl-buffered 20% D_2O saline) was measured at 40.25 MHz in the Fourier transform mode; 16 μsec r.f. pulse, 1.5 sec interpulse time, and 6000 transients. Key: (a) control rat; and (b) CCl_4 (2.0 ml/kg) administered rat. Arrows indicate the resonance of ortho-phosphoric acid as a standard.

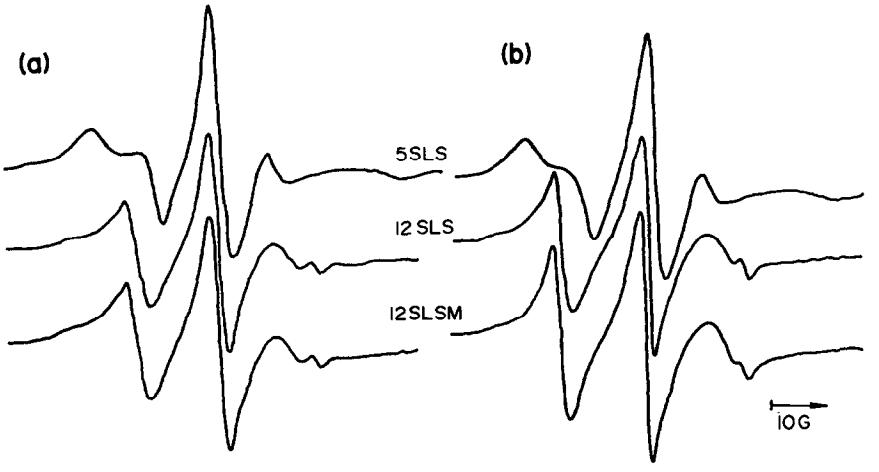

Fig. 2. Electron spin resonance spectra of 5SLS, 12SLS and 12SLSM in liver microsomes from control and from CCl_4-treated rat at 25°. Twenty nanomoles of each spin-probe were incubated with 50 μl of the microsomal suspension (13.3 mg protein/ml) for 1 min at room temperature and the spectrum was recorded immediately at 9.5 GHz. Key: (a) control rat; and (b) CCl_4 (2.0 ml/kg) administered rat.

measured at 37° with ^{31}P-NMR spectroscopy. Typical spectra are shown in Fig. 1. The spectrum from control rats showed an isotropic signal with a chemical shift of −2.0 ppm (ortho-phosphoric acid was used as a standard) and a peak width of *ca.* 30 ppm, which agrees with that reported by Stier *et al.* [7] and de Kruijff *et al.* [8, 9]. At 4°, a bilayer type signal was observed in agreement with that of Stier *et al.* [7] (data not shown). They suggested that the isotropic signal observed in microsomes may arise from "non-bilayer" structures in microsomal membranes. No appreciable change could be observed in the ^{31}P-NMR spectrum from samples taken during 26 hr following the oral administration of CCl_4 at a dose of 2 ml/kg (one-third of the LD_{50}) (Fig. 1). The results show that CCl_4 or its metabolites probably do not change the characteristic structure of liver microsomal membranes.

ESR measurements of membrane fluidity. Figure 2 shows the typical ESR spectra of rat liver microsomes with 5SLS, 12SLS, and 12SLSM. All spectra indicate that the spin-probes (5SLS, 12SLS, and 12SLSM) were incorporated into the membranes. 5SLS was undergoing an anisotropic rotation along the fatty acyl chain, whereas the spectra of 12SLS and 12SLSM indicate less ordering. No appreciable difference could be observed in the spectra of 5SLS between control and CCl_4-treated rats. The spectra of 12SLS or 12SLSM, however, were changed by the oral administration of CCl_4. The ratio of the peak height in the higher magnetic field [h(−1)] to the central peak height [h(0)] increased from treatment with CCl_4. To evaluate the effect of CCl_4 on the membrane fluidity, the order parameter of 5SLS and apparent rotational correlation time of 12SLS were calculated from the spectra shown in Fig. 2. The order parameter of 5SLS did not change, while the apparent rotational correlation time of 12SLS decreased from 4.0 nsec to 3.0 nsec by the oral administration of CCl_4 at a dose of 2 ml/kg (Table 1). The findings indicate that CCl_4 or its metabolites increase the fluidity of liver microsomal membranes, but the effect may be confined to the lipophilic region of the membranes.

Presence of two domains in microsomal membranes. The ESR spectrum of 5SLSM in rat liver microsomes significantly differed from those of the other three spin-probes (5SLS, 12SLS and 12SLSM). The spectrum comprised two different signals, a rather isotropic signal and an anisotropic one, and the ratio of the two signals depended on the incubation time (Fig. 3a). Immediately after the preparation of spin-labeled microsomes, a rather predominant isotropic signal was observed, but the intensity reduced gradually with the appearance of an anisotropic signal like the one of 5SLS; this reached a steady state within 60 min. We previously observed an ESR spectra consisting of two kinds of signals in liposomal membranes [21] and erythrocyte membranes [23]; one is an anisotropic signal in the lipid bilayers and the other is an isotropic signal in the aqueous medium. Comparison of the rather isotropic signal observed in microsomes with the isotropic signal in an aqueous medium, however, revealed a ratio of peak height [h(+1)/h(0)] that was quite different. The ratio in microsomes [h(+1)/h(0) = 0.6] was smaller than that in an aqueous medium [h(+1)/h(0) = 0.95], indicating that the rather isotropic signal in microsomes might arise from the spin-probes incorporated into membranes.

Several isosbestic points were observed in the spectra shown in Fig. 3a. This finding implies that the spin-probe 5SLSM should exist in two different states in microsomal membranes. 5SLSM may, at first, incorporate into a more fluid phase; in this phase 5SLSM can undergo isotropic tumbling similar to that of 12SLS or 12SLSM: this would give a rather isotropic ESR signal. During incubation, 5SLSM may transfer to a less fluid phase, resulting in the appearance of the anisotropic signal. According to this speculation, we calculated the ESR spectra of 5SLSM in microsomal membranes (Fig. 3c). The

Table 1. Effect of CCl_4 on fluidity of microsomal membranes*

	Order parameter (5SLS)	Apparent rotational correlation time (12SLS) (nsec)
Control	0.700 ± 0.001	4.0 ± 0.07
CCl_4	0.701 ± 0.001	3.0 ± 0.27

* Values are means ± S.D.

calculated spectra agreed with the spectra observed. A little disagreement was found between observed (Fig. 3a) and calculated spectra (Fig. 3c), especially at the lower magnetic field. A part of 5SLSM may be reduced in the experimental condition by nitroxide reductase which should be present in liver microsomes [12].

The oral administration of CCl_4 at a dose of 2 ml/kg, one-third of the LD_{50}, changed significantly the ESR spectrum of 5SLSM in rat liver microsomes. Only an isotropic signal could be observed; furthermore, this was independent of the incubation time (Fig. 3b). The findings suggest that the interaction of CCl_4 or its metabolites with the membranes may interfere with the transfer of 5SLSM from the fluid phase to the less fluid phase.

Effect of incubation temperature on 5SLSM distribution. To confirm this postulate described above, the ESR spectrum of 5SLSM in microsomes was measured at different temperatures (Fig. 4). In the control rat, only an anisotropic signal could be observed at 4°; a rather predominant isotropic signal was observed at temperatures higher than 18°. The spectral change by temperature was reversible, suggesting that the partial hydrolysis of 5SLSM to 5SLS may not be responsible for the spectral change in Fig. 3. The parallel component of the anisotropic signal increased at lower temperatures, e.g. from 26.4 G at 18° to 29.2 G at 4°. The larger parallel component of the hyperfine splitting at lower temperature indicates that the fluidity of the lipid bilayer of microsomal membranes should decrease at lower temperatures. Disappearance of a rather isotropic signal may relate to the low membrane fluidity. The membrane fluidity seems to be important in the distribution of 5SLSM between an isotropic state and an anisotropic state.

In the CCl_4-treated rat, an anisotropic signal in addition to an isotropic signal became observable by cooling to 4°, while above 15° no appreciable anisotropic signal could be observed (Fig. 4b). The spectrum of 5SLSM in the CCl_4-treated rat at 4° seems to be similar to that of control rats at 18°, indicating that CCl_4 or its metabolites might cause a dynamic change in microsomal membranes which lowers the equilibrium temperature in the distribution of 5SLSM between the two states.

Dose response of 5SLSM distribution. The partition of 5SLSM seems to be a sensitive index for membrane damage by CCl_4. To examine whether the partition of 5SLSM depends on the dose of CCl_4 or not, different amounts of CCl_4 were administered orally to rats. The ratio of the two signals, an anisotropic and a rather isotropic signal, depended on the dose of CCl_4 administered. The peak heights at lower magnetic field, which are indicated with ha and hi in Fig. 5, were measured for each signal and the ratio of ha to hi was plotted against the dose of CCl_4 (Fig. 5). The ratio decreased with increase of CCl_4

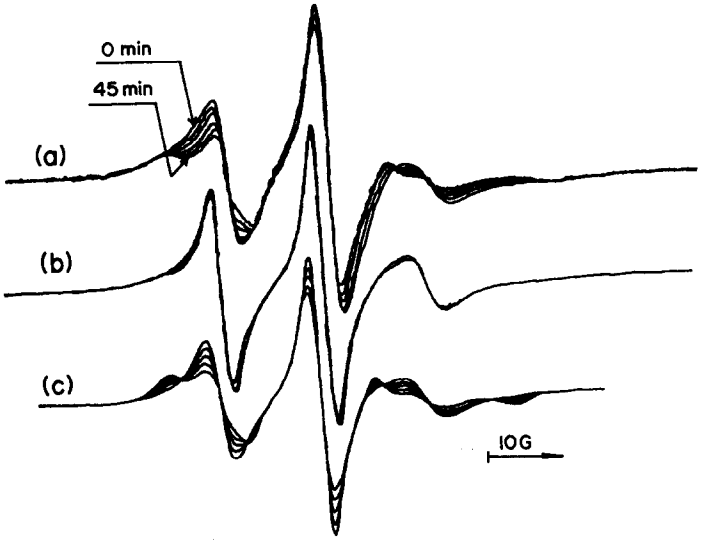

Fig. 3. Experimental and simulated electron spin resonance spectra of 5SLSM in rat liver microsomes. Details are described in the legend of Fig. 2. Key: (a) experimental spectra from control rat; (b) experimental spectra from CCl_4 (2.0 ml/kg) administered rat; and (c) simulated spectra. The simulated spectra were calculated using the combination of the anisotropic and isotropic models. The ESR line shape used was first derivative Lorentzian with peak-to-peak widths of 4.0 G and 2.0 G for the anisotropic signal and the isotropic one respectively.

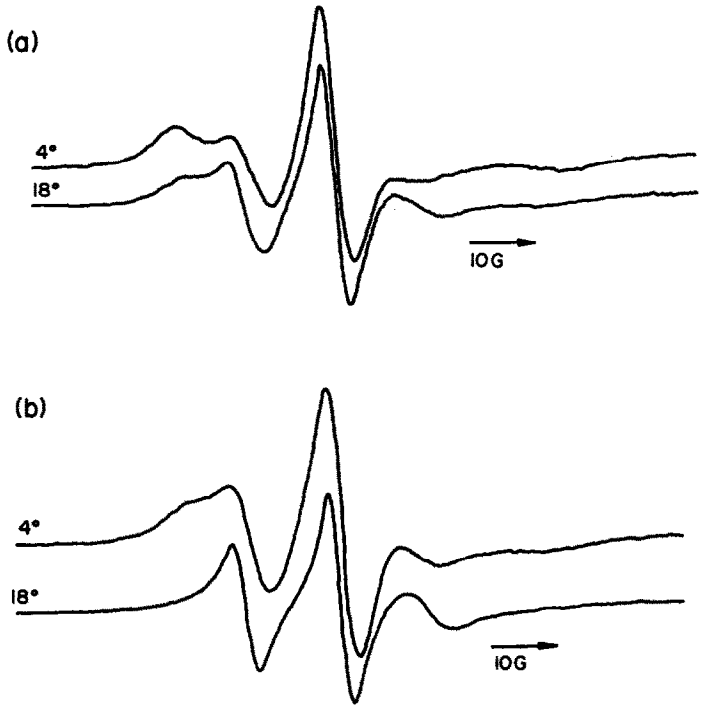

Fig. 4. Temperature dependence of the ESR spectra of 5SLSM in rat liver microsomes. Details are described in the legend of Fig. 2. Temperature was controlled with nitrogen stream and measured with a copper–constantan thermocouple. Key: (a) control rat; and (b) CCl_4-treated rat.

administered. The good dose response of the ratio suggests that partition of 5SLSM may be a sensitive index for membrane-damage induced by CCl_4. The *in vitro* addition of CCl_4 or $CHCl_3$ to control microsomes also decreased the ratio of the anisotropic signal to the isotropic one (data not shown).

Effect of membrane protein on 5SLSM distribution. The oral administration of CCl_4 leads to an elimination of some membrane-bound enzymes. To clarify the effect of membrane protein on the ESR spectrum, reconstituted microsomal vesicles with different amounts of protein were prepared. Figure 6 shows the relation between the partition of 5SLSM and the protein content in membranes. The ratio of the anisotropic signal to the isotropic one (ha/hi) decreased with a decrease of protein concentration.

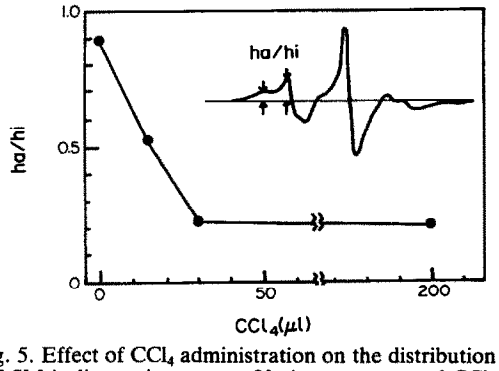

Fig. 5. Effect of CCl_4 administration on the distribution of 5SLSM in liver microsomes. Various amounts of CCl_4 in CMC-Na were orally administered to rats. After 3 hr, the spin-labeled microsomes with 5SLSM were prepared as described in Materials and Methods. At the time reached plateau, the peak heights, ha and hi (shown in the upper-right of this figure), were measured, and the ratio of ha/hi was plotted against the dose of CCl_4.

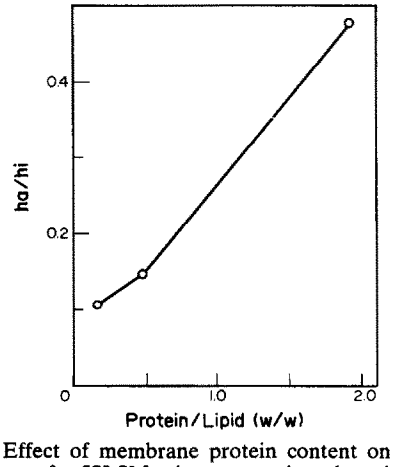

Fig. 6. Effect of membrane protein content on the distribution of 5SLSM in reconstituted microsomal membranes. Reconstitution was carried out with Triton X-100 and Bio Beads SM-2 as described in Materials and Methods. Reconstituted vesicles were labeled with 5SLSM, and the spectrum was recorded at room temperature. The peak heights, ha and hi, were measured as described in the legend of Fig. 5.

These findings indicate that the content of membrane protein may partially influence the distribution of 5SLSM in membranes; however, Triton X-100 and Bio-Beads SM-2 treatment should alter the membrane architecture.

DISCUSSION

In this paper, we studied, using ^{31}P-NMR spectroscopy and spin-labeling techniques, a structural change in rat liver microsomal membranes after the oral administration of carbon tetrachloride.

The ^{31}P-NMR spectrum of rat liver microsomes showed a rather isotropic signal at physiological temperature (37°) in accordance with the results of rabbit liver microsomes [7] and rat liver endoplasmic reticulum [8]. Stier *et al.* [7] suggested that a fraction of membrane lipids in rabbit liver microsomes may exist in an inverted micellar state "non-bilayer", which resulted in an isotropic signal. That spectrum strikingly depended on temperature and showed a bilayer shape at low temperature (8°). Similar results were also obtained for rat liver slices or intact perfused rat liver by de Kruijff *et al.* [9]. The findings indicate that the isotropic signal should not arise from the change of vesicle size. The ^{31}P-NMR spectrum of rat liver microsomes did not appreciably change after the oral administration of CCl_4. These results suggest that the characteristic structure of microsomal membranes containing an inverted micellar state might, on the whole, not be altered by either CCl_4 or its metabolites.

In contrast to the ^{31}P-NMR technique, the spin-labeling method can provide other information about microenvironmental mobility at different carbon positions of membrane lipids; this was done by using a series of spin-labeled stearic acids containing a nitroxide group at various carbon atoms. With the spin-probes we used, 5SLS gives motional information at the region relatively near the membrane surface, while 12SLS is useful for estimation of fluidity in the central domain of the membranes. The ESR spectrum of 5SLS and its order parameter were scarcely changed by the administration of CCl_4. This dynamic property at the region near the membrane surface should, therefore, be unaffected by CCl_4 administration, in agreement with the results of ^{31}P-NMR.

On the other hand, the spectra of 12SLS and 12SLSM were changed by CCl_4 administration. This implies that a segmental motion near the center of lipid bilayers was increased by CCl_4 or its metabolites. For the index of the segmental motion, apparent rotational correlation times were calculated from the ratio of peak height at the higher magnetic field to the central peak height as described by Keith *et al.* [22] on the basis of the equation by Kivelson [24]. As pointed out by Cannon *et al.* [25], Kivelson's equation is based on the assumption of free tumbling motion. The spectrum of 12SLS was relatively isotropic, but the motion of 12SLS should be asymmetric in membranes. Therefore, we must note that the rotational correlation times obtained from the spectra of 12SLS are apparent values. The decrease of apparent rotational correlation time (Table 1) indicates that CCl_4 or its metabolites increase the microenvironmental mobility around the carbon-12 position of membrane lipids. The action of CCl_4 in microsomes is believed to be peroxidation triggered by trichloromethyl radical, the metabolite of CCl_4; this may cause a structural change in membranes through lipid–lipid and lipid–protein cross-links [5]. If so, the structural change by cross-linking should be more marked at the region near the center of membranes.

The ESR spectrum of 5SLSM (Fig. 3) in microsomes was rather different from that of 5SLS (Fig. 2). The spectrum of 5SLSM comprised two signals, a rather isotropic and an anisotropic one. The ratio of the anisotropic signal to the isotropic one depended on the incubation temperature, the protein–lipid ratio in membranes, and the dose of CCl_4. With decreasing temperature, the amount of a rather isotropic signal decreased in conjunction with the appearance of an anisotropic signal which arose from the spin probe in the lipid bilayer. From ^{31}P-NMR analysis, de Kruijff *et al.* [9] suggested that the temperature-dependent "bilayer" $\rightleftarrows$ "isotropic" transition should occur in microsomal membranes and that the fraction of the ^{31}P-NMR signal with a "bilayer" line shape may increase from 35% at 30° to 90% at 4°. If the rather isotropic ESR signal relates to the occurrence of the isotropic ^{31}P-NMR signal, 5SLSM can easily incorporate into non-bilayer structures because of the higher lipophilicity of the methyl ester than that of the free fatty acid.

The presence of protein in membranes, in general, alters the dynamic properties of membranes [26, 27]. The depletion of membrane proteins from microsomes increased the intensity of the rather isotropic signal (Fig. 6). Liposomal membranes consisting of total microsomal lipids showed a "bilayer" structure in ^{31}P-NMR spectra [9]. The addition of cytochrome *c* seems to induce "non-bilayer" structures in cardiolipin-containing liposomal membranes [28]. In this experiment, the ratio of protein to lipid was not reduced significantly by CCl_4 administration (data not shown). CCl_4 administration might, therefore, affect the lateral distribution of proteins in microsomal membranes and a protein-poor domain may be produced in the membranes.

With increasing dose of CCl_4, the ratio of the anisotropic signal to the rather isotropic one decreased clearly in the ESR spectrum of 5SLSM in microsomes (Fig. 5). The rather isotropic signal also increased by adding CCl_4 directly to liver microsomes. We have not determined the residual CCl_4 in microsomes but some of CCl_4 administered was reported to remain relatively long in microsomes [29]. Therefore, it should not be neglected that the change of ESR spectra may be due to the physical presence of CCl_4. Ortner *et al.* [30] also observed that the rather isotropic signal of 5SLSM was more predominant in mastocytoma cells that in rat mast cells. Therefore, the distribution of 5SLSM in microsomal membranes may be affected by a small change in membrane architecture. 5SLSM seems to be a very suitable probe for the estimation of membrane damage.

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