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Effect of "Drugs for Liver Disease" on Hepatotoxic Action of Carbon Tetrachloride. III.[1] Effect of Protoporphyrin and Phosphorylcholine on Injured Microsomal Membrane[2]

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An attempt has been made to clarify the action of drugs for liver disease, protoporphyrin (PP) and phosphorylcholine (PC) on the structure of liver microsomal membrane during carbon tetrachloride (CCl_4) intoxication, by measuring ultraviolet (UV) absorbance, infrared (IR) spectroscopy, circular dichroism (CD) and ribonucleic acid (RNA) content, and by using some hydrophobic probes, 1-anilinonaphthalene-8-sulfonate (ANS) and 2,4,6-trinitrobenzenesulfonate (TNBS). Administration of PC to CCl_4-poisoned rats was found to decrease the increased nonribosomal RNA content of the microsomes at 5 days, to some extent. ANS binding to microsomes little changed in all groups. A double reciplocal plot of ANS binding to the microsomes indicated that the affinity of the membrane of all groups for ANS was not affected by CCl_4 or the drug administrations. TNBS binding to the aminophospholipids, phosphatidylserine (PS) and phosphatidylethanolamine (PE), however, on the membrane was extremely decreased in CCl_4-poisoned rats at 2 and 5 days, by 21—25% for PS and 25—34% for PE, as compared with those of control rats, indicating a significant alteration of the phospholipid composition in the membrane. Administration of PC for 8 days produced a significant increase in its binding to both aminophospholipids. It was found from CD spectra of the membrane that CCl_4 administered caused partially conformational changes of the proteins, significant at 5 days, and administration of PC to the poisoned rats for 5 days recovered the decreased ordered structure to the native level. A semigraphical method of CD data analysis exhibited that the membrane contained approximately 55% α-helix, 21% β-structure and 24% unordered structure and CCl_4 administration decreased the helix content by 9% without significant alteration of β-structure. IR spectra also indicated that the membrane contained β-structure. PP had no appreciable effects on the structure of the membrane. Thus, it is concluded that PC has an excellent reconstructive action of phospholipid and protein structures of the injured microsomal membrane.

It has been reported that carbon tetrachloride (CCl_4) administered *in vivo* binds to liver microsomal lipids thus leading the lipid peroxidation[4] and primarily affects the endoplasmic reticulum membrane, followed by inactivation of many proteins,[5] including cytochrome P-450 (P-450).[6] The solvent is also found to cause the alteration of fatty acid compositions of microsomal phospholipids.[7] An abundance of recent evidence indicates that the properties of many microsomal enzymes are intimately related to interactions with the microsomal phospholipids.[8] However, a detailed study on the alteration of the microsomal membrane

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structure in injured liver and on the effect of drugs for liver disease on the structure has remained unclear.

We have been studing on the effects of some drugs for liver disease on lysosomal enzymes[9] and microsomal drug-metabolizing enzymes[1] in rat liver, in an attempt to clarify the mechanism of their actions. As a result of the studies, it was found that protoporphyrin administered protected lipid peroxidation and induced microsomal drug metabolizing enzymes, while phosphorylcholine produced the increase in microsomal phospholipid content in the injured liver. This paper is concerned with studying the effect of drugs on liver microsomal membrane structure during CCl_4 intoxication, using a hydrophobic fluorescent probe, 1-anilinonaphthalene-8-sulfonate (ANS)[10] and a non-penetrating probe, 2,4,6-trinitrobenzenesulfonate (TNBS),[11] infrared (IR) spectroscopy and circular dichroism (CD).

Experimental

Matarials——Protoporphyrin disodium salt (PP) and phosphorylcholine chloride calcium salt (PC) were a gift from Dozin Iyakukako. ANS and TNBS were purchased from Tokyo Kasei Kogyo Co., Ltd.

Animals——Male Wistar rats, weighing 100 to 130 g, maintained on MF diets (Oriental Yeast Co., Ltd.) for 3—4 days prior to the experiment, were employed throughout the study. The animals were divided at random into 3 groups, each consisting of 4—5 rats, and fasted for 12 hr prior to the experiment. A) Controls, treated for 2 days with daily *s. c.* injections of 0.2 ml of olive oil per 100 g of body weight followed by a daily oral administration of water (Control-rats). B) Animals, treated for 2 days with daily *s. c.* injections of a mixture of 0.2 ml of CCl_4 and 0.2 ml of olive oil per 100 g of body weight followed by a daily oral administration of PP (0.75 mg/100 g body weight) or PC (12.5 mg/100 g body weight) solution (PP-rats or PC-rats). C) Animals, treated as described in B), except that water was given instead of the drug solution (CCl_4-rats).

The animals were treated by the same method as described in the previous paper,[1] except that the doses of the drugs to rats were 5-fold the clinically effective daily doses.

Preparation of Liver Microsomal Fractions——The liver microsomal fractions were prepared according to the method of Omura and Sato.[12] For the removal of trapped, soluble proteins from the microsomal preparations the method of Kamat and Wallach was used.[13] The washed microsomes were finally suspended in isotonic NaCl phosphate buffer (NaCl 18.0 g, NaH_2PO_4 0.374 g and Na_2HPO_4 2.731 g/2 liters, pH 7.4) and used for the measurements of CD, ultraviolet (UV) and IR spectra. For determinations of ribonucleic acid (RNA) and ANS and TNBS binding, the microsomal fractions were prepared in 0.15 M KCl containing 25 mM Tris-HCl buffer, pH 7.4 and 10 mM EDTA for the purpose of removing ribosomes.[14]

Measurements of CD Spectra——CD spectra of the microsomal fractions were measured in a JASCO J-20 automatic recording spectropolarimeter with a cuvette of 0.1 mm-optical path. The microsomal suspensions were diluted with isotonic NaCl phosphate buffer to give a concentration of about 1 mg of protein per ml.

Measurements of UV Spectra——UV spectra of microsomal fractions were measured in a Shimadzu MPS-50L spectrophotometer, using a quartz cell of 1.0 cm-optical path. The microsomal suspensions were diluted with 0.1 M phosphate buffer, pH 7.0, to give a concentration of 0.1 mg protein per ml. The absorpance at 260 nm was corrected for that of protein, although it was very small.

Measurements of IR Spectra——A JASCO IRA-1 grating infrared spectrophotometer was used. Solid films were prepared by applying about 1.0 mg membrane protein (in aqueous suspension) on the polyethylene film and drying in vacuum for 2—3 days.

Measurements of ANS-microsome Fluorescence——5×10^{-5} M ANS, dissolved in 0.1 M phosphate buffer, pH 7.4, were added to an equal volume of the microsomal suspensions (0.3 mg of protein per ml of 0.1 M phosphate buffer, pH 7.4) in a quartz cell of 1.0 cm-optical path and the fluorescence of ANS associated with the microsomal membrane was measured in a Shimadzu RF-501 recording spectrofluorophotometer. The excitation wavelength was held constant at 380 nm and the emission wavelength was 470 nm. The experiment on a double reciprocal plot of ANS binding to the microsomes was carried out at the concentrations of 1.5—20×10^{-6} M ANS in the medium.

TNBS Binding to Aminophospholipids——TNBS binding to aminophospholipids was carried out according to the method of Bonsall and Hunt[15] with a slight modification. Two ml of the microsomal fraction was incu-

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bated with 2.0 ml of 0.1% TNBS dissolved in 0.1 M sodium phosphate buffer, pH 8.0, for 24 hr at room temperature, and then 7 volumes of a 1: 1 mixture of chloroform: methanol were added to the mixture and mixed for 30 sec. After centrifugation at 1500 × *g* for 10 min the lower phase was evaporated to dryness and the remained lipid were dissolved in the chloroform–methanol mixture. Individual phospholipid class was separated by thin-layer chromatography (TLC) on Kieselgel 60 HR (Merck) in a solvent system of chloroform–methanol–water (130: 50: 7, by volume). The ninhydrin reaction to estimate the extent of reaction of PS and PE was carried out by spraying the TLC plates with the ninhydrin solution, according to the procedure described by Gordesky and Marinetti.[11] The results were expressed as the percentage of total aminophospholipid.

Separation and Determination of RNA——The isolation of the nucleic acid from the microsomes was performed according to the method of Schneider.[16] RNA contents were measured using Bial's reaction which determines the pentose component in the nucleic acid[17] with RNA from Torula yeast (Sigma Chemical Co.) as the standard.

Protein Determination——Protein concentration was determined by the procedure described by Lowry, *et al.*[18] with bovine serum albumin, fraction V, as a standard.

Results

UV Absorbance and RNA Content

The UV absorbance of liver microsomal vesicles, removed soluble proteins by osmotic shock of 0.01M Tris-HCl buffer, pH 8.6,[13,19] was measured at 260 nm. As shown in Table I, the absorbance which is probably resulted from the nucleic acid of ribosomes was enhanced in CCl_4-rats by 21% and in PP-rats by 26% at 2 days as compared with Control-rats, while at 8 days the absorbance of all groups was decreased nearly to the control value. This indicates that RNA content in the microsomes injured by CCl_4 administered was restored within 8 days under this condition. On the other hand, the absorbance in PC-rats was lesser than that of CCl_4-rats and was almost the same as that of Control-rats, indicating that PC appears to contribute indirectly to decrease in the increased UV absorbent substances after incorporation to the membrane.

The RNA contents of the microsomes removed ribosomes with 10 mM EDTA[14] are shown in Table II. As a result, the amount of nonribosomal RNA which is tightly bound to microsomal membrane was found to increase in CCl_4-rats more than in Control-rats, especially significant at 2 and 5 days. In PC-rats, the increased content was decreased nearly to control

Table I. UV Absorbance of Liver Microsomal Membranes from Rats treated with CCl_4 and Drugs

Rats	OD_{260}/mg protein (days)		
	2	5	8
Control-rats	5.0±0.7	4.8±0.4	4.9±0.6
PP-rats	6.3±0.4[a]	5.7±0.4[a]	5.0±0.7
CCl_4-rats	6.1±0.4[b]	5.4±0.3	5.4±0.1
Control-rats	4.4±0.3	4.2±0.1	4.4±0.1
PC-rats	4.6±0.1	4.6±0.3	4.4±0.1
CCl_4-rats	5.1±0.1[c]	4.9±0.2[c]	4.8±0.1

Each value represents the mean of 4—5 rats ± standard error.
a) $p<0.05$ in Control-rats *vs.* PP-rats
b) $p<0.05$ in Control-rats *vs.* CCl_4-rats
c) $p<0.02$ in Control-rats *vs.* CCl_4-rats

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18) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
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Table II. RNA Contents of Microsomes, Removed Ribosomes, treated with CCl_4 and Drugs

Rats	RNA (μg/mg protein) (days)		
	2	5	8
Control-rats	196.7± 5.6	195.9±16.0	194.4± 7.9
PP-rats	214.4±20.2	244.0±17.5[a]	213.3± 3.9
CCl_4-rats	217.2±14.6	247.5±28.1[b]	196.0± 9.0
Control-rats	191.5± 8.0	196.2± 8.3	181.4±10.9
PC-rats	218.0± 5.4[c]	218.8± 3.7[c]	181.9±22.5
CCl_4-rats	225.7±13.4[d]	242.5± 6.8[e]	190.0±11.3

Each value represents the mean of 4—5 rats ± standard error.
a) $p<0.02$ in Control-rats *vs.* PP-rats
b) $p<0.02$ in Control-rats *vs.* CCl_4-rats
c) $p<0.01$ in Control-rats *vs.* PC-rats
d) $p<0.01$ in Control-rats *vs.* CCl_4-rats
e) $p<0.02$ in PC-rats *vs.* CCl_4-rats

level at 5 days, suggesting that PC has a restoring action to the native RNA content in the membrane. The administration of PP, however, was found not to decrease the component enhanced by CCl_4 administration. This result agreed nearly to that of the UV absorbance.

ANS-microsome Fluorescence

ANS, a hydrophobic fluorescent probe,[10] was adapted to the microsomal fractions to study the membrane conformational changes. ANS binding to the microsomes was not changed in all groups at 2—8 days, suggesting that the organization of the hydrophobic region in the membrane was not affected by administration of CCl_4 and the drugs (5.9—7.8 fluororescence intensity/mg protein). A double reciplocal plot of ANS binding to the microsomes as a function of ANS concentration is shown in Fig. 1. It is obvious that the change of the affinity of the membrane for ANS was not induced by the treatments. The apparent dissociation constant (K_{app}) for Control-rats was 2.7—3.9×10^{-5}M, for CCl_4-rats 3.2—3.8×10^{-5}M, for PP-rats 3.0—4.2×10^{-5}M and for PC-rats 2.4—4.2×10^{-5}M at all days.

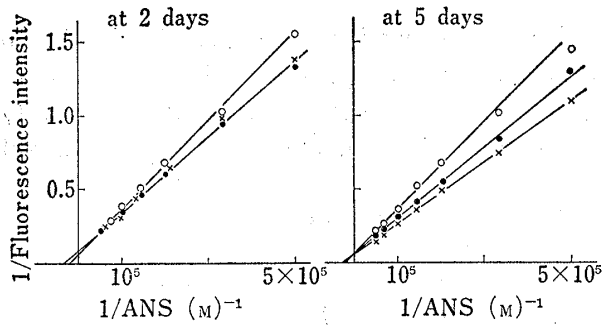

Fig. 1. Double Reciplocal Plots of ANS Binding to Microsomes treated with CCl_4 and Drugs

ANS concentrations were 1.5×10^{-6}M to 2.0×10^{-5}M in the medium.
●: Control-rats ○: PC-rats ×: CCl_4-rats

TNBS Binding to Aminophospholipids

The binding of TNBS, which is impermeable to the biological membrane and easily binds to amine groups of phospholipids and proteins,[15] was estimated in order to clarify the arrangement of some phospholipid species on the microsomal membrane. Phosphatidylserine (PS) and phosphatidylethanolamine (PE) were separated by TLC and the extent of reaction of TNBS with the phospholipids was estimated by ninhydrin reaction. The data shown in Tables III and IV indicate that about 81—85% of the total PE and 53—61% of the total PS reacted with the reagent in Control-rats and the greater part of the PE localized on the outer surface of the membrane. In CCl_4-poisoned rats and PC-rats the amount of the PE and PS reacted with the reagent was significantly decreased at 2 and 5 days, suggesting that CCl_4 administration caused an extreme alteration of the phospholipid composition on the membrane surface. However, administration of PC for 8 days produced an increase in the

decreased phospholipids, PE and PS, on the membrane surface in contrast to a further decrease at 2 and 5 days. The significant increase of these phospholipids at 8 days is probably due to the formation of PE and PS from diglyceride increased.[20]

Table III. TNBS Reaction with Phosphatidylserine on Microsomes, Removed Ribosomes, treated with CCl_4 and Drugs

Rats	Phosphatidylserine reacted with TNBS (%) (days)		
	2	5	8
Control-rats	55.0±4.2	55.1±7.0	57.7±4.7
PP-rats	35.2±6.4[a]	38.9±6.3[a]	46.8±6.6
CCl_4-rats	30.9±5.3[b]	33.1±6.6[b]	40.1±4.5[b]
Control-rats	60.5±3.0	54.6±2.0	58.7±6.1
PC-rats	38.3±6.1[c]	22.6±7.2[c]	54.1±7.0
CCl_4-rats	36.0±7.3[d]	31.8±2.8[b,e]	43.6±4.4[b,e]

Each value represents the mean of 4—5 rats±standard error.
a) $p<0.02$ in Control-rats *vs.* PP-rats
b) $p<0.02$ in Control-rats *vs.* CCl_4-rats
c) $p<0.05$ in Control-rats *vs.* PC-rats
d) $p<0.05$ in Control-rats *vs.* CCl_4-rats
e) $p<0.01$ in PC-rats *vs.* CCl_4-rats

Table IV. TNBS Reaction with Phosphatidylethanolamine on Microsomes, Removed Ribosomes, treated with CCl_4 and Drugs

Rats	Phosphatidylethanolamine reacted with TNBS (%) (days)		
	2	5	8
Control-rats	82.9±1.8	84.0±3.7	83.8±3.5
PP-rats	51.5±7.3[a]	56.9±3.1[a]	68.7±2.3[a]
CCl_4-rats	50.9±6.1[b]	59.2±6.1[b]	68.8±2.1[b]
Control-rats	84.6±5.0	81.4±2.3	82.6±1.8
PC-rats	65.4±6.7[a]	38.2±6.6[a]	73.5±3.9[c]
CCl_4-rats	51.1±5.1[b,d]	50.3±7.9[b,d]	66.2±2.2[b,d]

Each value represents the mean of 4—5 rats±standard error.
a) $p<0.01$ in Control-rats *vs.* PP-rats or PC-rats
b) $p<0.01$ in Control-rats *vs.* CCl_4-rats
c) $p<0.05$ in Control-rats *vs.* PC-tars
d) $p<0.01$ in PC-rats *vs.* CCl_4-rats
e) $p<0.05$ in PC-rats *vs* CCl_4-rats

IR Spectra

Fig. 2 shows IR spectra of solid films of liver microsomal membrane at 2 days. The membrane had an amide I band at 1650 cm^{-1} and amide II band at 1545 cm^{-1}. The peak at 1720—1750 cm^{-1} due to C=O stretching in fatty acid esters was found to increase in CCl_4-rats, probably ascribed to increase in neutral lipids. The small negative peaks at 1532, 1635 and 1680 cm^{-1} which are characteristic of β-structure of protein[21] were observed in the membrane. This suggests that the microsomal proteins contain β-structure in addition to α-helix and unordered structure, as demonstrated with other membranes such as mitochondria.[22]

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22) J.M. Graham and D.F.H. Wallach, *Biochim. Biophys. Acta*, **193**, 225 (1969).

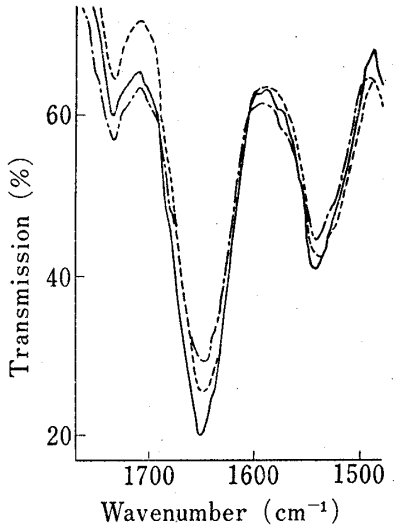

Fig. 2. Infrared Spectra of Microsomal Membranes treated with CCl_4 and PP

————: Control-rats,
————: PP-rats,
----------: CCl_4-rats

CD Spectra

CD spectra of the microsomal membrane of all groups at 2, 5 and 8 days are shown in Fig. 3. The data gave the change in ellipticity of the minima at about 222 nm in CCl_4-rats at 5 days, as shown in Table V, while at 2 and 8 days only small changes are produced. The ellipticity at 222 nm for CCl_4-rats at 5 days decreased by 8% as compared with Control-rats, indicating that CCl_4 administered causes partially conformational changes of the membrane proteins, including membrane-bound enzymes. The ellipticity for PC-rats, however, was the same as that of Control-rats. This also suggests that PC contributes the reformation of the injured membrane proteins. On the other hand, in PP-rats at 5 days no restoration of α-helix content was obtained, although at 8 days the decreased content partially recovered, suggesting that PP administered did not act on the reformation of the injured membrane proteins. These findings permit one to draw certain assumption about the nature of the microsomal membrane that the breakdown of the membrane proteins by CCl_4 administration occurs far later than peroxidative damage of the lipids, which damage is observed several hours after administration of CCl_4,[6a] and the decrease in the helical content may be partially due to the damage of protein syntheses by ribosomes in early stage of CCl_4 intoxication and thus lead to the decrease in drug-metabolizing enzymes tightly bound to the membrane.

The CD spectrum of Control-rats exhibits the minima at 208 and 222 nm characteristic of α-helical conformation, as shown in Fig. 4. A semigraphical method of CD data analysis proposed by Greenfield and Fasman[23] was applied to the figure and the fitted values calculated from poly-L-lysine spectra were plotted. The simulated plots, which were obtained by shifting each point in the membrane curve towards the blue by 3 nm, at wavelengths from 212—250 nm were made taking 55% α-helix, 21% β-structure and 24% unordered structure for the

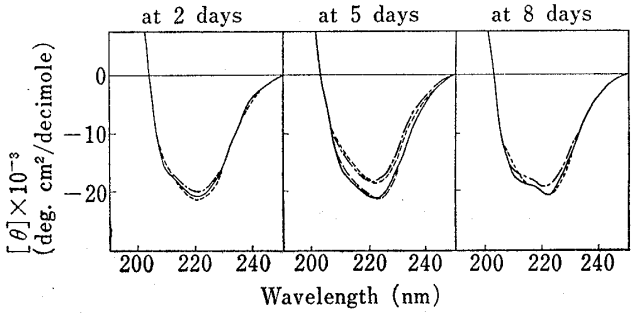

Fig. 3. Far-ultraviolet CD Spectra of Microsomal Membranes treated with CCl_4 and Drugs

The microsomes were homogenized before the measurement using a glass homogenizer.
————: Control-rats, ----------: PP-rats,
- - - - - -: PC-rats, ————: CCl_4-rats

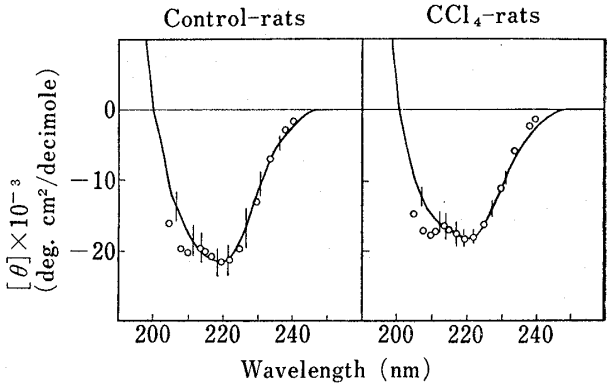

Fig. 4. Far-ultraviolet CD Spectra of Microsomal Membranes of Control- and CCl_4-rats at 5 days

Solid curve, experimental result; open circles, (left) simulated values with 55% α-helix, 21% β-structure and 24% unordered structure, (right) 46% α-helix, 22% β-structure and 32% unordered structure, calculated from poly-L-lysine.

23) N. Greenfield and G.D. Fasman, *Biochemistry*, **8**, 4108 (1969).

TABLE V. The Values of $[\theta]_{222}$ and α-Helical Composition of Microsomal Membranes treated with CCl_4 and Drugs

Days	Rats	$[\theta]_{222}$	α-Helix (%)	Rats	$[\theta]_{222}$	α-Helix (%)
2	Control-rats	−21193±1125	53.0±2.8	Control-rats	−22764± 379	56.9±1.0
	PP-rats	−21758±1116	54.4±2.8	PC-rats	−22567± 803	56.4±2.0
	CCl_4-rats	−20611±1335	51.5±3.3	CCl_4-rats	−21825± 280	54.6±0.7
5	Control-rats	−21476±1720	53.7±4.3	Control-rats	−22901± 572	57.3±1.4
	PP-rats	−18199± 457	45.5±1.1[a]	PC-rats	−22842±1171	57.1±2.9
	CCl_4-rats	−18101± 471	45.3±1.2[b]	CCl_4-rats	−19846± 706	49.6±1.8[b,c]
8	Control-rats	−20934± 652	52.3±1.6	Control-rats	−22422± 256	56.0±0.6
	PP-rats	−20413± 924	51.0±2.3	PC-rats	−22452± 371	56.1±0.9
	CCl_4-rats	−19655±2349	49.1±5.9	CCl_4-rats	−21420± 406	53.5±1.0

Microsomes were adjusted to a protein concentration of 1.0 mg per ml. The amount of α-structure was calculated by following approximate relations, $f_H=[\theta]_{222}/40000$

a) $p<0.05$ in Control-rats *vs.* PP-rats

b) $p<0.05$ in Control-rats *vs.* CCl_4-rats

c) $p<0.05$ in PC-rats *vs.* CCl_4-rats

microsomal membrane of the rat liver. This analysis proves that there is β-structure in the membrane. On the other hand, the best fit to the experimental curve of CCl_4-rats at 5 days is obtained with 46% α-helix, 22% β-structure and 32% unordered structure, therefore, a 9% decrease of α-helix content was recognized. This indicates that CCl_4 administered causes only partial loss of ordered secondary structure (α-helix) of the proteins. Of particular interest was that the β-structure of the membrane remained unaltered in spite of autocatalytic, peroxidative breakdown of the helical structure during CCl_4 intoxication.

Discussion

It is assumed that CCl_4 administered is activated to a free radical form by interaction with a normally occurring homolytic process under metabolic control in liver microsomal fractions.[24] The free radicals formed then interact with neighbouring lipid-rich material and increase lipid peroxidation, causing disturbance in structure and function.[24] The structure of the lipid phase in microsomal membranes has been postulated to be an absolute requirement for the activities of several enzymes which are bound tightly to the membrane[8b,25] P-450, the terminal oxidase in drug oxidation,[26,27] is located in the hydrophobic region of the membrane[28] and the microsomal NADPH-cytochrome c reductase, when catalyzing the reduction of P-450, requires phospholipid for activity.[29] The decrease in the reduction rate of P-450 in the liver of CCl_4-treated animals is assumed to result in damaging unsaturated fatty acids of phosphatidylcholine in the process of peroxidation.[30] At the same time Sugano *et al.* have demonstrated a decrease in the phosphatidylcholine content of the microsomes of CCl_4-poisoned animals.[31] On the other hand the conformation of the proteins of microsomal membrane and interrelationships between membrane lipids and proteins during liver injury are not well understood.

Drugs for liver disease, PP and PC, have indeed proven to be clinically effective for liver diseases. The mechanism of these action, however, is only partially clarified. To clarify

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the action of these drugs on the structure and function of liver microsomal membrane during liver injury, some experiments have been initiated.

In the present study, ANS binding to the microsomes was found not to be affected by CCl_4 or the drug administrations. Since there are several findings to suggest that ANS-microsome fluorescence is largely attributable to ANS binding to membrane phospholipids,[10,30] the results obtained indicate that the hydrophobic environment of the microsomal membrane is little altered during CCl_4 intoxication. A similar conclusion has been reached by Archakov and Karuzina in the process of CCl_4-poisoning.[30] A double reciprocal plot of the ANS binding to the microsomes also showed that the affinity of the membrane for ANS did not change in all groups. These results suggest that the overall organization of the membrane lipids are not changed by free radicals and lipid peroxides and the extent of hydrophobic contacts between proteins and lipids is unaltered on the membrane surface during CCl_4 intoxication, although there may be a difference in the phospholipid composition in the membrane. TNBS binding to aminophospholipids on the membrane surface, however, was decreased by about 25% in CCl_4-rats at 2 days as compared with that of Control-rats (Tables III and IV), suggesting that there was a significant alteration of the phospholipid composition on the membrane surface of the endoplasmic reticulum in CCl_4 intoxication. This decrease is probably due to destruction of these phospholipids by free radicals and peroxides, because Jacob and Lux have found that PE in erythrocyte membrane is destroyed during peroxidation.[32] When PC is administered to the poisoned rats for 8 days, a significant increase in TNBS binding, decreased by CCl_4 intoxication, are observed. This indicates that PC is incorporated into the membrane and then contributes the reconstruction of these phospholipids. An extreme decrease of TNBS binding to PE and PS on the microsomal surface in PC-rats at 5 days may be due to the increase in phosphatidylcholine on the surface based on the incorporation of the compound into the membrane and masking of PE. The incorporation of PC into the membrane for 8 days probably enhances the biosyntheses of phospholipids in the membrane and renews a resulting recovery of the functions and structures. Since the structure of the lipid phase in microsomal membrane is an absolute requirement for some drug-metabolizing enzymes,[28,29] these changes in the arrangement of phospholipids appear to cause secondarily both qualitative and quan titative changes of the enzyme activities.

It was found that RNA content of the microsomal membrane was increased by CCl_4 administration. A definite explanation for this phenomenon is difficult on the basis of the result obtained. However, this increase may be related to the changes in the membrane components, since it has been suggested that the endoplasmic reticulum contains about 3—4% of RNA tightly bound to the membrane.[33]

The CD spectra of the microsomal membrane indicated that the ellipticity at 222 nm for CCl_4-rats at 5 days was decreased by 8% as compared with Control-rats (Table V), whereas it is recovered by PC administration. This suggests that CCl_4 administration causes the partially conformational changes in the membrane proteins, and that PC incorporated into the membrane acts to restore the secondary structure of the membrane protein. The effect of PC may be due to an indirect action that PC induces the rearrangement of the phospholipids of the membrane and leads to normalize hydrophobic interactions between lipids and proteins, suggesting an importance of the hydrophobic interactions for the function of the membrane. These suggestions are supported by the proposal that loss of PE in certain localities in erythrocyte membrane during peroxidation causes aggregation of residual hydrophobic membrane proteins.[32] The CD spectrum of the microsomal membrane of the normal rat liver exhibited that the membrane contains approximately 55% α-helix, 21% β-structure and 24% unordered structure (Fig. 4). Thus, it would appear that the high α-helical protein content measured

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in this experiment is a general property of most membrane proteins.[34,35] The presence of β-structure in the membrane is also demonstrated by the data of IR spectroscopy (Fig. 2). Recently, the presence of β-conformation in the mitochondrial membrane[22,35] and rat adipocyte plasma membrane[36] has been shown by IR spectroscopy. It is of interesting that CCl_4 administered caused a partial loss of α-helix of the membrane protein without significant alteration of β-structure, probably at least partially, leading the disturbance of hydrophobic interactions with phospholipids. The contribution of the protein conformation, however, to the function and structure of microsomes is the subject for a future study.

One important possibility that the state of the microsomes which are fragments of endoplasmic reticulum, separating from the cell may partly differ from that of the endoplasmic reticulum *in vivo* should be noted in this study. Despite the qualified nature of such a study, it seems possible, nevertheless, to draw many limited conclusions from the results of this study on the microsomes.

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