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CCl_4-INDUCED DAMAGE TO ENDOPLASMATIC RETICULUM MEMBRANES

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Abstract—Cytochrome P-450 can be used to indicate damage of the membranes of endoplasmatic reticulum by CCl_4. Free radicals are formed during the metabolism of CCl_4, which stimulate peroxidation of unsaturated fatty acids and directly inactivate cytochrome P-450. The first indication of cytochrome P-450 inactivation is a decrease in the reduction rate in the NADPH-specific electron transfer chain. In microsomes obtained from the liver of poisoned animals NADPH-cytochrome P-450 reductase is not stimulated by DMA.

NUMEROUS investigations have shown that CCl_4 primarily affects the membranes of the endoplasmatic reticulum,[1–4] this process being accompanied by the inactivation of many proteins, including cytochrome P-450.[5–10]

Hemoprotein was used as a marker for the effect of CCl_4 on the membranes of endoplasmatic reticulum. An attempt was made to discover if the free radical products of CCl_4 metabolism directly affected the membranes of endoplasmatic reticulum, or if its damaging effect was due to the pro-oxidating action of its metabolites. Kinetic parameters which characterized cytochrome P-450 participation in the reactions of hydroxylation were measured in microsomes isolated from normal liver (controls) and from the liver of animals treated with CCl_4.

EXPERIMENTAL

Male rats weighing 200–250 g fed *ad lib.* were used in these studies. In experiments *in vivo*, CCl_4 was injected i.p. (0·6 ml per 100 g body wt). One hr after administration the animals were decapitated. A microsomal fraction was prepared as described previously.[11,12]

In experiments *in vitro* the glucose-6-phosphate dehydrogenase system was used to maintain a constant level of reduced NADP. The glucose-6-phosphate dehydrogenase system contained: 8 mM $MgCl_2$; 50 mM nicotinamide; 40 mM Tris–HCl buffer (pH 7·4); 9 mM glucose-6-phosphate Na; glucose-6-phosphate dehydrogenase 0·5 U/ml (Koch–Light, England). The reaction was initiated by adding 0·3 mM NADP,* the microsomal protein content being 10 mg/ml. Five μl of CCl_4 per 2 ml of the mixture were added to the side tube of a Warburg vessel. A small amount was taken off as a sample after incubation at 37° with constant shaking, and was used for analysis.

* Abbreviations used: DMA, dimethylaniline; UFA, unsaturated fatty acid; FA, formaldehyde; MDA, malonic dialdehyde; NDPO, NADPH-dependent peroxidation of UFA; ADPO, ascorbate-dependent peroxidation of UFA; ANS^-, 1-anilinonaphthalene-8-sulphonate.

The rates of hydroxylation for DMA and UFA peroxidation were determined by the quantity of FA and MDA formed.[5,11,12]

The content of cytochrome P-450 was measured on a Hitachi-356 double wavelength, dual beam spectrophotometer. Three ml of the incubation mixture contained 250 mM Tris–HCl buffer (pH 7·5) and ≈ 5 mg of microsomal protein. CO was blown through the cuvette for 1 min and then several mg $Na_2S_2O_4$ were added. The measurement was made at $\Delta A_{450-490}$.

The reduction rate of cytochrome P-450 was measured in anaerobic conditions. Three ml of the incubation medium contained 40 mM Tris–HCl buffer (pH 7·4) and ≈ 2·5–3·0 mg of microsomal protein. Submitochondrial particles* (≈ 2 mg of protein), succinate and rotenone at a final concentration of 15 mM and 2·0 μM, respectively were added to the incubation mixture to ensure the maintenance of anaerobic conditions. Before measurement, CO was blown through the incubation mixture for 1 min. The reaction was initiated by adding 40 μM of NADPH to the incubation medium with the help of a special device for rapid mixing. The measurement was made in a Hitachi 356 spectrophotometer. The reaction rate was calculated as described by Gigon *et al.*[10]

Spectral changes, arising with the interaction of DMA with cytochrome P-450 were recorded on a Hitachi 356 spectrophotometer.[13]

K_m, V_{max}, K_s and $\Delta A_{max385-425}$ were calculated according to Lineweaver–Burk.[14]

ANS^- fluorescence was recorded on a EF-3M fluorimeter using glass filters (transmission maximum at 366 nm and recording a transmittance maximum at 470–480 nm). The incubation medium contained 50 mM Tris–HCl buffer (pH 7·4), 1 mg of protein and ANS^- in a concentration of 5–25 μM. In experiments with ANS^- the microsomal fraction was prepared in a medium containing 0·15 per cent KCl, 10 mM EDTA and 25 mM Tris–HCl buffer (pH 7·4). EDTA was added to the isolation medium to inhibit peroxidation of UFA and to dissociate ribosomes.

RESULTS AND DISCUSSION

Studying the effect of CCl_4 on the rate of formation of lipid peroxides in microsomes, we have shown that the effect of the pro-oxidation action of CCl_4 is dependent on the concentration of microsomal protein (Table 1). It can be clearly seen from this data that acceleration of the formation of MDA in the presence of CCl_4 takes place only when the content of microsomal protein in the sample is greater than 8 mg/ml. At lower concentrations the effect is negligible.

The effect of pro-oxidation action of CCl_4 in the presence of NADPH results in free radical products being formed in the processes of CCl_4 metabolism. To further this hypothesis the reactions of stimulated peroxidation of CCl_4 in the presence of EDTA was studied. This suggested that addition of EDTA (0·5–1·0 mM) to the incubation mixture may block the formation of MDA as a result of binding of the Fe^{2+} ions which generate free radicals initiating peroxidation reactions of UFA. The same concentrations of the complexon cause only an insignificant effect on the hydroxylation rate of DMA (Fig. 1). Hence, in these conditions the rate of metabolism of CCl_4 should not change drastically. When CCl_4 was added to the incubation mixture,

* Submitochondrial particles obtained from beef heart were kindly given us by the Bioenergetics Department, Laboratory of Bio-organic Chemistry, Moscow State University.

TABLE 1. EFFECT OF THE MICROSOMAL PROTEIN CONTENT ON THE VALUE OF PROOXIDATION ACTION OF CCl_4*

Exp. conditions†	Incubation time (min)	Content of microsomal protein (mg/ml) 2	4	8
Control	15	12·1	11·7	9·2
	30	18·9	19·0	14·5
$+CCl_4$	15	13·4	14·4	15·9
	30	17·4	19·7	20·8
Difference	15	+1·3	+2·7	+6·7
	30	−1·5	+0·5	+6·3

* Results are expressed as nanomoles MDA/milligram protein and the averages of three experiments.

† The incubation mixture contained: 40 mM Tris–HCl buffer (pH 7·4), NADPH-generating glucose-6-phosphatedehydrogenase system and a different quantity of microsomal protein.

containing 0·6 mM EDTA, the pro-oxidant action of the radicals, formed in the course of CCl_4 metabolism, could be clearly seen (Table 2). The absence of such an effect in the system containing ascorbate instead of NADPH, leads to the conclusion that the mechanism of the pro-oxidant action of CCl_4 is in fact the formation of free radicals. Thus from the data given above we may conclude that free radicals are formed by the metabolism of CCl_4.

The mechanism of inactivation of cytochrome P-450 in damaged membranes was studied measuring peroxidation stimulated by NADPH with/without CCl_4.

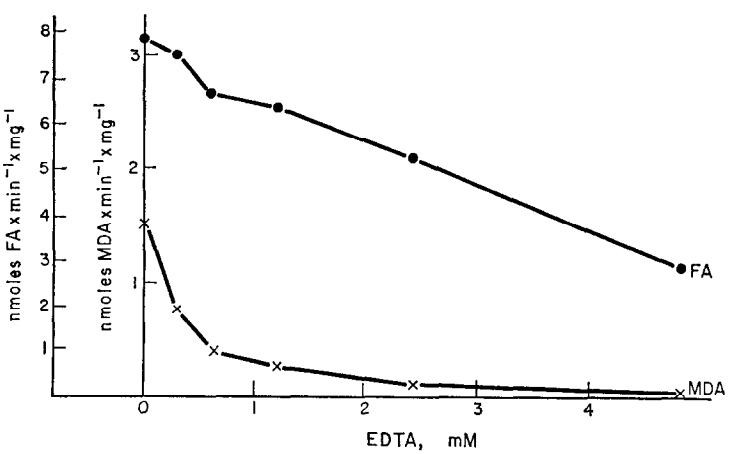

FIG. 1. Inhibition of the DMA hydroxylation reaction and peroxidation of UFA by EDTA. The rate of DMA demethylation was measurement by the quantity of the FA formed. Incubation media contained: 40 mM Tris–HCl buffer (pH 7·4); 16 mM $MgCl_2$; 3 mM NADPH; 6 mM DMA; 2 mg of the microsomal protein. Total volume of the incubation media is 1 ml. The rate of UFA peroxidation was measurement by the quantity of the MDA formed. Incubation media contained: 25 mM Tris–HCl buffer (pH 7·4); 0·012 mM Fe^{2+}; 0·2 mM NaPP; 3 mM NADPH; 0·5–1 mg of the microsomal protein.

TABLE 2. EFFECT OF CCl_4 AND EDTA ON FORMATION RATE OF MDA IN NDPO AND ADPO*

Exp. conditions†	Incubation time (min)	NDPO	ADPO
Control	15	9·0	12·0
	30	16·4	21·4
+0·6 mM EDTA	15	0·4	0·2
	30	0·5	0·3
+0·6 mM EDTA	15	5·3	0·2
$+CCl_4$	30	7·5	0·5

* Results are expressed as nanomoles MDA/milligram protein and the averages of four experiments.

† NADPH-generating system was used in NDPO and ascorbate-dependent system (40 mM Tris–HCl buffer, pH 7·4; 0·012 mM Fe^{2+} and 0·2 mM NaPP; 0·8 mM ascorbate) was used in ADPO.

The data obtained were expressed as nanomoles of inactivated cytochrome P-450/1 μmole of the lipid peroxides formed (measured as MDA in both systems). The results (Table 3) show that the damaging activity of peroxides is much higher if the incubation medium contains CCl_4. This effect can be explained by the action of an additional quantity of radical products, formed as a result of CCl_4 metabolism. Thus we may conclude that at least with respect to cytochrome P-450, its inactivation involves free radicals from CCl_4 and not only the processes of peroxidation of UFA of membrane lipids.

TABLE 3. INACTIVATING EFFECT OF CCl_4 METABOLITES ON CYTOCHROME P-450*

Incubation time (min)	No. of exp.	NADPH-generating system	NADPH-generating system + CCl_4	P
10	5	25·8	36·6	< 0·025
20	9	19·5	32·7	< 0·005
40	8	19·8	25·5	< 0·001

* Results are expressed as nanomoles of cytochrome P-450 per 1 μmole of MDA.

To elucidate the mechanism of inactivation of cytochrome P-450, molecular properties of the hemoprotein, characterizing its participation in hydroxylation reactions, was studied. A kinetic analysis of the hydroxylation reaction was made with microsomal preparations from control and CCl_4-treated animals. The K_m value for DMA in the control and experimental preparations of microsomes is the same (Table 4, Fig. 2). A decrease in V_{max} in microsomes after addition of CCl_4 (calculated per 1 mg of protein) can be explained by a decrease in the cytochrome P-450 content in them (30 per cent, Table 4) because a part of the hemoprotein converts to P-420 (Fig. 3). No decrease in V_{max} when calculated per nanomole of hemoprotein (k_{+2}) proves that neither K_m nor V_{max} are changed in the cytochrome P-450 left (Table 4).

TABLE 4. THE CHANGE OF THE MOLECULAR PROPERTIES OF CYTOCHROME P-450 IN MICROSOMES POISONED WITH CCl_4*

	Hydroxylation				Spectral analysis	
	V_{max} (nmoles FA.min^{-1}.mg^{-1})	k_{+2}‡ (min^{-1})	K_m (mM)	Cyt. P-450 (nmoles.mg^{-1})	$\Delta A_{385-425}$ (cm^{-1}.mg^{-1})	K_s (mM)
Control	6·9	7·4	0·48	0·9	0·0051	0·56
Experiment†	4·6	7·0	0·49	0·7	0·0049	1·12
P	< 0·01			< 0·01		< 0·01

* Results are expressed as averages of five experiments.

† Microsomal fraction was obtained from animals poisoned with CCl_4, which was injected i.p. in doses 0·6 ml/100 g of animal weight 1 hr before decapitation.

‡ k_{+2} min^{-1} are calculated as V_{max} of the hydroxylation reaction per nmole of cytochrome P-450.

A study of the spectral properties of cytochrome P-450 showed an increase in the spectral binding constant of hemoprotein in microsomal preparations which were obtained from animals injected with CCl_4 (Table 4, Fig. 4). The absence of identity changes of K_m and K_s can be explained by the fact that K_m does not reflect the affinity of cytochrome P-450 for DMA as the value k_{+2}/k_{+1} is rather large. At the same time an increase in K_s was not accompanied by a change in the character of binding; the maximal amplitude of the spectral changes during DMA binding ($\Delta A_{385-425}$) is the same in the normal and affected liver.

The measurement of the reduction rate of cytochrome P-450 showed that a decrease in the rate of cytochrome P-450 reductase takes place. It can be clearly seen in Table 5 and Fig. 5 that both the initial reduction rate of cytochrome P-450 (V_0) and the appearance rate constant of microsomes in the control animals was much higher (2–3 times).

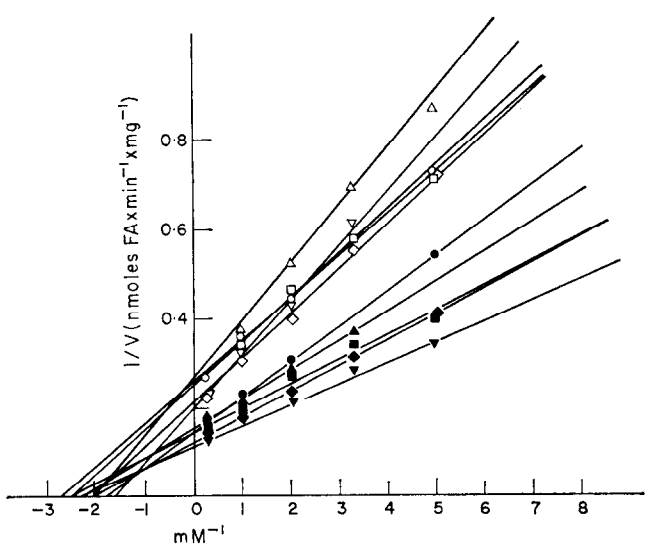

FIG. 2. Effect of CCl_4-poisoning on K_m and V_{max} hydroxylation reaction of DMA.
Legends: (●——●); (▲——▲); (◆——◆); (■——■); (▼——▼)—control,
(○——○); (△——△); (◇——◇); (□——□); (▽——▽)—experiment.

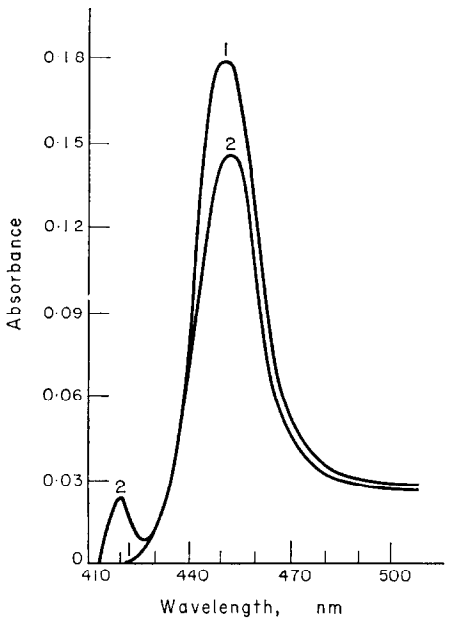

FIG. 3. Difference spectra of cytochrome P-450 in normal conditions and when poisoned by CCl_4.
Legends: 1—control; 2—experiment.
The incubation mixture contained: 40 mM Tris–HCl buffer (pH 7·4), 6 mg of microsomal protein in 3 ml mixture. The mixture of one of the cuvette CO was blown through for 1 min. Then mixtures of both cuvettes were reduced by several mg of $Na_2S_2O_4$. The record was made on a Hitachi 356 double beam spectrophotometer.

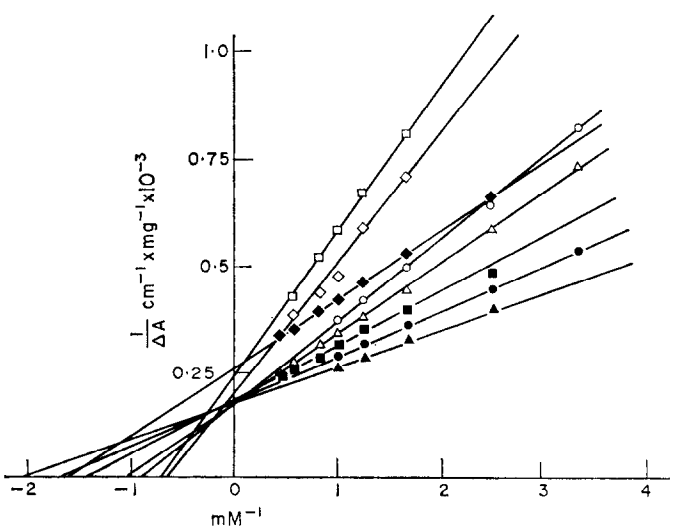

FIG. 4. Effect of CCl_4-poisoning on K_s and maximal amplitude of the spectral changes ($\Delta A_{385-425}$) of cytochrome P-450.
Legends: (▲——▲); (●——●); (■——■); (◆——◆)—control,
(△——△); (○——○); (□——□); (◇——◇)—experiment.
The incubation mixture contained 40 mM Tris–HCl buffer (pH 7·4), 10 mg of microsomal protein in 3 ml mixture.

TABLE 5. CHANGES IN THE REDUCTION RATE OF CYTOCHROME P-450 IN CCl_4 POISONED MICROSOMES*

	Without DMA		+ 0·8 mM DMA	
	V_0 (nmoles.min^{-1}.mg^{-1})	k_{+2} (min^{-1})	V_0 (nmoles.min^{-1}.mg^{-1})	k_{+2} (min^{-1})
Control	3·2	3·5	4·9	5·4
Experiment†	1·4	2·0	1·5	2·1
P	< 0·01		< 0·01	

* Results are expressed as averages of seven experiments.
† Experimental conditions are the same as those in Table 4.

An interesting fact in NADPH-cytochrome P-450 reductase is that microsomes damaged with CCl_4, are not stimulated by DMA, this is in contrast to the controls, where this stimulation is quite significant (1·5 times).

These results show changes in cytochrome P-450 in the liver of CCl_4-treated animals, which result in a decrease in its reducibility by NADPH-cytochrome P-450 reductase.

In our previous work[5] and that of other authors[15–17] it has been shown that the damaging effect of CCl_4 on cytochrome P-450 *in vitro* is in agreement with its pro-oxidant effect. A decrease in the content of cytochrome P-450 takes place only when the content of MDA in the incubation media increases. The use of antioxidants in the given systems causes a more pronounced protecting action than addition of glutathione. While studying the change in the molecular properties of cytochrome P-450 in the systems forming lipid peroxides, but not containing CCl_4, we concluded that they are similar to the changes observed in animals dosed with CCl_4 (Table 6,

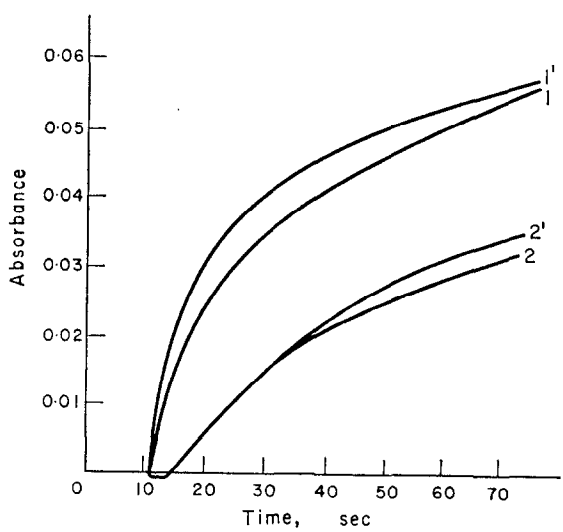

FIG. 5. The change of the reduction rate of cytochrome P-450 at CCl_4-poisoning.
Legends: control preparations: 1—without DMA, 1′—with 0·8 mM of DMA, experimental preparations: 2—without DMA, 2′—with 0·8 mM DMA.

TABLE 6. EFFECT OF PEROXIDATION OF UFA ON K_m AND V_{max} REACTIONS OF HYDROXYLATION OF DMA*

	V_{max} (nmoles FA.min^{-1}.mg^{-1})	K_m (mM)
Control	4·9	0·66
Experiment†	2·9	0·65
P	< 0·01	

* Results are expressed as averages of four experiments.
† Experimental conditions see to Fig. 6.

Fig. 6). As can be seen from the table, the microsomal preparations, where peroxidation was stimulated by the addition of NADPH and FePP, a considerable decrease in V_{max} took place (calculated per milligram of protein) whereas the K_m value remains unchanged in the *in vitro* experiments (Table 4). Hence there should be little difference between the mechanism of inactivation of cytochrome P-450 *in vivo* due to the action of CCl_4 and *in vitro* where peroxidation is stimulated by NADPH and FePP.

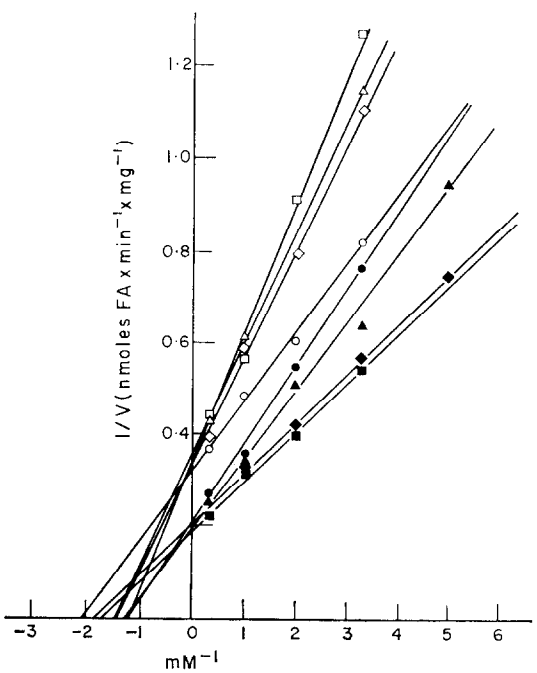

FIG. 6. Effect of stimulation of the processes of peroxidation of UFA on K_m and V_{max} in hydroxylation reaction of DMA in microsomes.

Legends: (●——●); (▲——▲); (◆——◆); (■——■)—control,
(○——○); (△——△); (◇——◇); (□——□)—experiment.

Control incubation mixture contained all the components of glucose-6-phosphatedehydrogenase system excluding $NADP^+$. In experimental samples: 0·3 mM $NADP^+$, 0·012 mM FePP, 0·2 mM NaPP was added to it. Mixtures were incubated at 37° for 30 min. Then 0·25 ml of the sample was taken off and was used for determination of DMA hydroxylation rate.

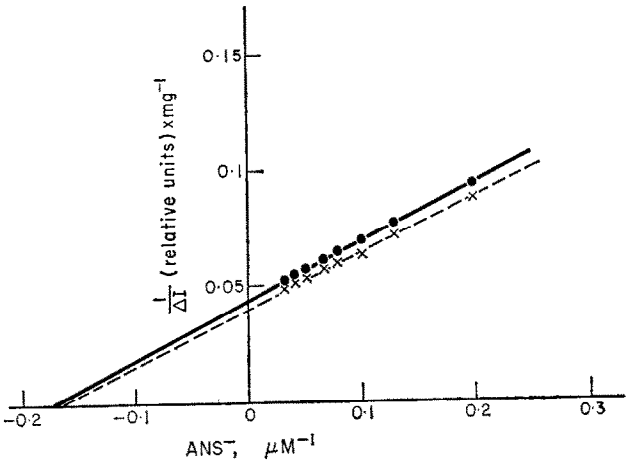

FIG. 7. Binding of ANS^- by rat liver microsomes in control preparation (×——×) and at CCl_4-poisoning (●——●). I—intensivity of ANS^- fluorescence.

It was assumed that the decrease in the reduction rate of cytochrome P-450 resulted in damaging UFA of phosphatidylcholine in the process of peroxidation. Coon *et al.*[18] showed that the reduction rate of cytochrome P-450 in the reconstructed systems depends on the presence of the given phospholipid in the mixture. At the same time authors have demonstrated a decrease in the phosphatidylcholine content of the microsomes of animals poisoned with CCl_4.[19]

To clarify further whether the hydrophobic environment of choline groups of phospholipid is in fact poisoned by CCl_4, ANS^- was used, which according to the data of Eling and DiAugustine,[20] binds mainly with this phospholipid in the membranes of the endoplasmatic reticulum.

As can be seen from Fig. 7, neither the number of binding places of ANS^- (K_s = 5·85 μM) nor its hydrophobic environment change considerably in the process of CCl_4-poisoning. The value of the fluorescence maximal yield per milligram of protein of the poisoned animals did not differ from that of the control ($\Delta I_{cont.} = \Delta I_{exper.} = 25$ relative units).

CONCLUSION

The damaging effect of CCl_4 on the cytochrome P-450 results in the formation of free radical products in the process of metabolism in the membranes of the endoplasmatic reticulum. Free radical products formed are responsible for two kinds of effects. Firstly, they stimulate peroxidation of UFA of the membrane lipids and, secondly they produce a direct damaging effect on cytochrome P-450. Inactivation of cytochrome P-450 takes place which was expressed in a decrease in V_{max} calculated per milligram of microsomal protein. Cytochrome P-450 whose spectral characteristics remain unchanged has the same V_{max} and K_m as hemoprotein in the intact microsomes. At the same time it is reduced much less effectively by NADPH-cytochrome P-450 reductase. Thus the earliest signs of damage to cytochrome P-450 due to CCl_4 are a decrease in its reduction rate in the NADPH-specific electron transfer chain and the

absence of a stimulating effect of DMA on the rate of this reaction. The binding of ANS^- with membranes of the endoplasmatic reticulum is not changed by CCl_4-poisoning.

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