Mechanism of Inhibition by Carbonyl Cyanide *m*-Chlorophenylhydrazone and Sodium Deoxycholate of Cytochrome *P*-450-Catalysed Hepatic Microsomal Drug Metabolism

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1. Treatment of liver microsomal fraction with 0.03–0.12% sodium deoxycholate and 0.005–0.06 mM carbonyl cyanide *m*-chlorophenylhydrazone decreases phospholipid-dependent hydrophobicity of the microsomal membrane, assayed by the kinetics of 8-anilidonaphthalene-1-sulphonate binding and ethyl isocyanide difference spectra. 2. Sodium deoxycholate at a concentration of 0.01% lacks its detergent properties, but competitively inhibits aminopyrine binding and activates the initial rate of NADPH-cytochrome *P*-450 reductase. In the presence of 0.03–0.09% sodium deoxycholate the rate-limiting factor in *p*-hydroxylation of aniline is the content of cytochrome *P*-450, and that for *N*-demethylation of aminopyrine is the activity of NADPH-cytochrome *P*-450 reductase. 3. Carbonyl cyanide *m*-chlorophenylhydrazone has no effect on the binding and metabolism of aniline; investigation of its inhibiting effect on aminopyrine *N*-demethylase established that the rate-limiting reaction is the dissociation of the enzyme–substrate complex in the microsomal preparations. 4. In the mechanism of action of carbonyl cyanide *m*-chlorophenylhydrazone the key step may be the electrostatic interaction of its protonated form and one of the forms of activated oxygen at the catalytic centre of cytochrome *P*-450. 5. At least two different phospholipid-dependent hydrophobic zones are assumed to exist in the microsomal membrane, both coupled with cytochrome *P*-450. One of them reveals selective sensitivity to the protonation action of carbonyl cyanide *m*-chlorophenylhydrazone and contains the ‘binding protein’ for type I substrates and NADPH-cytochrome *P*-450 reductase; the other contains the cytochrome *P*-450 haem group and binding sites for type II substrates.

The problems of regulating the enzymic activity in multicomponent membrane-bound systems have currently become of interest because of the elucidation of not only the structural, but also the functional role for the lipid component of biomembranes. The essential requirement of phospholipid for microsomal cytochrome *P*-450 activity in the mono-oxygenase system is, in principle, characterized by three criteria, postulated by Fleischer *et al.* (1962), namely, decrease in the enzymic activity on removal of phospholipid (Omura & Sato, 1964; Chaplin & Mannering, 1970; Eling & DiAugustine, 1971; Leibman & Estabrook, 1971), re-activation of the enzyme on addition of phospholipid (Cater *et al.*, 1972; Lu & Levin, 1974; Vore *et al.*, 1974) and association of enzyme with phospholipids (Omura & Sato, 1964; Ichikawa & Yamano, 1967; Imai & Sato, 1967; Leibman & Estabrook, 1971).

Treatment leading to alterations of the phospholipid component of the microsomal membrane changes the binding and metabolism of type I and type

II substrates (Schenkman *et al.*, 1967) in various ways (Shoeman *et al.*, 1969; Chaplin & Mannering, 1970; Leibman & Estabrook, 1971; Eling & DiAugustine, 1971; Tsyrllov & Lyakhovich, 1975), although it is known that phospholipids are essential for the hydroxylation of microsomal substrates of both types (Imai & Sato, 1960; Lu & Levin, 1974). On the basis of the data of Schenkman *et al.* (1967) and Remmer *et al.* (1968) that type I substrates bind in the lipoprotein moiety of cytochrome *P*-450 enzyme and that type II substrates affect the ligand of haem iron with the formation of ferrihaemochrome, it may be assumed that the sterically separated active centres of mono-oxygenase for type I and type II substrates are in association with different hydrophobic zones in the microsomal membrane.

We have attempted to determine the dependence of the various functional aspects of the microsomal mono-oxygenase system on its hydrophobic environment. This has been achieved with the aid of carbonyl cyanide *m*-chlorophenylhydrazone and

sodium deoxycholate, agents different in chemical structure but both affecting hydrophobic interactions in artificial and natural membranes (Hemker, 1962; Omura & Sato, 1964; Victoria & Barber, 1969; Verma *et al.*, 1973). We also intended to clarify the nature of the influence of these agents on the spectral and kinetic constants of the microsomal cytochrome *P*-450-containing mono-oxygenase.

These studies were presented in part at the 5th International Symposium on Drugs Affecting Lipid Metabolism, Milan, September 1974 (Tsyrllov & Lyakhovich, 1974) and at the 558th Meeting of the Biochemical Society, Edinburgh, September 1975 (Tsyrllov *et al.*, 1975).

Materials and Methods

All procedures for the preparation of the microsomal fraction from male Wistar (150–180 g) rat livers were as previously described (Tsyrllov & Lyakhovich, 1975; Tsyrllov *et al.*, 1976).

Aminopyrine *N*-demethylase activity was determined essentially as described by Smuckler *et al.* (1967). The incubation medium contained microsomal fraction (5–6 mg of protein), 0.1 M- $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer (pH 7.5), 5 mM-aminopyrine, 0.3 mM- NADP^+ and a NADPH-generating system consisting of 7 mM-glucose 6-phosphate, 10 mM- MgCl_2 and 3 units of glucose 6-phosphate dehydrogenase (EC 1.1.1.49). Total volume of the incubation medium was 3 ml. The amount of formaldehyde formed was measured by the procedure of Nash (1953).

Aniline *p*-hydroxylase activity was measured by the method of Imai *et al.* (1966). The incubation medium contained in final volume of 2 ml the microsomal fraction (6 mg of protein), 0.1 M-Tris/acetic acid buffer (pH 8.0), 8 mM-aniline, 0.32 mM- NADP^+ and NADPH-generating system as described above. The amount of *p*-aminophenol formed was measured at 630 nm, by using *p*-aminophenol as standard.

The amounts of cytochromes *P*-450 and *P*-420 were determined from CO-difference spectra of $\text{Na}_2\text{S}_2\text{O}_4$ -treated microsomal fraction as described by Omura & Sato (1964), by using $\epsilon = 91$ and $111 \text{ litre} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$ for cytochromes *P*-450 and *P*-420 respectively.

Ethyl isocyanide difference spectra were measured as described by Imai & Siekevitz (1971). Microsomal fraction (3.0 mg of protein/ml) in 0.2 M-potassium phosphate buffer at designated pH values, and 30% (v/v) glycerol were placed in both sample and reference cuvettes. Ethyl isocyanide (final concentration 0.4 mM) was added to sample cuvette and minimal amounts of $\text{Na}_2\text{S}_2\text{O}_4$ were added to both cuvettes. Scanning was started at 600 nm 3 min after $\text{Na}_2\text{S}_2\text{O}_4$ addition. The magnitudes of the 455 and 430 nm peaks of the ethyl isocyanide difference spectra of reduced cytochrome *P*-450 were expressed in terms of

$\Delta E_{455(430)-500}/\text{nmol}$ of cytochrome *P*-450, and the E_{455}/E_{430} ratio was calculated.

Difference spectra were recorded in a Hitachi model 356 two-wavelength double-beam scanning spectrophotometer, in the split-beam mode.

The activity of NADPH-cytochrome *c* reductase was determined kinetically under conditions similar to those of Phillips & Langdon (1962). Results were expressed as nmol of cytochrome reduced/min per mg of protein; ϵ was taken as $19.1 \text{ litre} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$ (King, 1967).

The activity of NADPH-cytochrome *P*-450 reductase was measured as described by Gnosspelius *et al.* (1969). Only the first linear segment (rapid-reaction phase) was used to calculate the rate of reduction, conventionally assuming ϵ for cytochrome *P*-450 to be $91 \text{ litre} \cdot \text{mmol}^{-1} \cdot \text{cm}^{-1}$. The effect of the substrate on the initial reaction rate was studied in this system, and also in the system containing 3.2 mM-aminopyrine from the start. The results were expressed in nmol of cytochrome reduced/min per mg of protein. Assays for the reductases were performed with a Hitachi-356 apparatus in the dual-wavelength mode.

The binding spectra of aminopyrine, a type I substrate, and aniline, a type II substrate, were determined by using microsomal fraction (5 mg of protein) suspended in 0.1 M- $\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4$ buffer (pH 7.5). Various increasing amounts of the substrates were added to the sample cuvette, the reference cuvette receiving equivalent volumes of solvent. The effects of carbonyl cyanide *m*-chlorophenylhydrazide and sodium deoxycholate on the substrate-induced spectra were measured under the same conditions. Thereafter equal quantities of the agents were added to both cuvettes. The spectral dissociation constant (K_s) for both type I and type II substrates was determined by the method of Schenkman *et al.* (1967). Maximal binding of the substrate to cytochrome *P*-450 (constant ΔE_{max}) was measured as described by Kato *et al.* (1971).

The fluorescence of 8-anilinoanthracene-1-sulphonate in the presence of the microsomal fraction was measured in a Spekol spectrofluorimeter. The incubation medium contained microsomal suspension at a concentration of 1.3 mg of protein/ml, 0.125 M-KCl, 0.02 M-Tris/HCl buffer (pH 7.4) and 8-anilinoanthracene-1-sulphonate at concentrations of 1–30 μM in a total volume of 2.5 ml. In studies of the intensity of 8-anilinoanthracene-1-sulphonate fluorescence in a medium containing control and experimental microsomal fractions of various protein concentrations (0.04–0.24 mg of protein) the concentration of the dye was 10 μM . Excitation was at 360 nm and emission was at 470 nm.

For protein determination the biuret method of Gornall *et al.* (1949) was used. Details of the experiments are in the legends to the Figures and Tables.

Results

Spectral characteristics of cytochrome P-450 and the activity of NADPH-dependent reductases

As shown in Fig. 1(a), addition of increasing concentrations of sodium deoxycholate to the sample cuvette caused a sequential, but significant ($P < 0.05$), decrease in the peak at 450 nm in $\text{Na}_2\text{S}_2\text{O}_4$ -reduced CO-bubbled microsomal suspension (see also Table 2). There was no conversion of cytochrome P-450 into cytochrome P-420, an enzymically inactive form, in the presence of sodium deoxycholate until the concentration of this agent reached 0.09%, which shows the absence of a stoichiometric relationship between the disappearance of cytochrome P-450 and appearance of cytochrome P-420.

On the other hand, the presence of increasing concentrations of carbonyl cyanide *m*-chlorophenylhydrazone was not accompanied by any changes whatever in the cytochrome P-450 spectrum (Fig. 1b) under various incubation conditions.

Investigation of the effect of these agents on the rate of NADPH-cytochrome *c* reductase has shown that carbonyl cyanide *m*-chlorophenylhydrazone did not alter the activity of this enzyme, whereas sodium

deoxycholate at concentrations of 0.03–0.09% somewhat increased the reaction velocity (Table 1).

Quite different effects were observed for NADPH-cytochrome P-450 reductase. As shown in Table 1, carbonyl cyanide *m*-chlorophenylhydrazone suppressed the initial phase of reductase reaction, the decrease in reductase activity being most pronounced when carbonyl cyanide *m*-chlorophenylhydrazone concentration reached $60\text{ }\mu\text{M}$ (39% of the control value); further increase in the amount of the agent did not change the reductase action. It is noteworthy that carbonyl cyanide *m*-chlorophenylhydrazone, although inhibiting NADPH-cytochrome P-450 reductase, did not remove the stimulating effect caused by the presence of type I substrate, such as aminopyrine.

As for sodium deoxycholate, this agent had a bi-phasic effect on the rate of NADPH-dependent reduction of cytochrome P-450. Thus 0.01% sodium deoxycholate stimulated reductase activity, but inhibited the same increase in the enzyme activity caused by aminopyrine (Table 1). These effects of 0.01% sodium deoxycholate could be explained by the fact that this derivative of cholesterol is a type I substrate for cytochrome P-450 and is characterized

Fig. 1. Difference spectra of CO-binding haemoprotein in rat liver microsomal fraction treated with sodium deoxycholate (a) and carbonyl cyanide *m*-chlorophenylhydrazone (b)

Incubation medium contained 0.125 M-KCl and 0.02 M-Tris/HCl buffer (pH 7.4) and microsomal fraction (1.9 mg of protein/ml). The measurements were made at 22°C. Assays were done (a) in the absence (—) and in the presence of 0.01% (·····), 0.03% (----), 0.06% (— —), 0.09% (— · —) and 0.12% (— · · —) sodium deoxycholate (b), and in the absence (—) and in the presence of $90\text{ }\mu\text{M}$ -carbonyl cyanide *m*-chlorophenylhydrazone (·····). Cytochrome P-450 content in untreated microsomal fraction was 0.92 ± 0.01 nmol/mg of protein. The result is the mean $\pm$ S.E.M. of five to eight tests.

Table 1. *Effects of carbonyl cyanide *m*-chlorophenylhydrazone and sodium deoxycholate on the activity of NADPH-dependent reductases in rat liver microsomal fraction*

Experimental details are given in the Materials and Methods section. Activities of reductases are expressed as nmol of reduced acceptor formed/min per mg of microsomal protein. Δ is the calculated stimulation of the initial rate of cytochrome *P*-450 reduction by addition of 3.2 mM-aminopyrine. All the values given are the means of five or six determinations, the results of which differed by less than 10%. n.d., Not detectable.

Carbonyl cyanide <i>m</i> -chloro-phenylhydrazone concn. (μ M)	NADPH-cytochrome <i>c</i> reductase		NADPH-cytochrome <i>P</i> -450 reductase					
	(% of control)		Without substrate		With 3.2 mM-aminopyrine			
			(% of control)		(% of control)		(% of control)	
0 (control)	93.3	100	2.0	100	2.6	100		30
10	93.0	100	1.84	92	2.34	90		28
20	93.0	100	1.8	90	2.36	90		31
30	91.4	99	1.4	70	1.8	69		29
60	93.7	100	1.22	61	1.59	61		30
90	90.9	97	1.22	61	1.55	60		27
Sodium deoxycholate concn. (%)								
0 (control)	86.8	100	1.8	100	2.39	100		33
0.01	86.6	100	2.25	125	2.25	94		0
0.03	90.1	104	1.43	80	1.6	67		12
0.06	91.3	105	0.68	35	0.63	26		0
0.09	92.2	106	0.35	20	0.35	15		0
0.12	86.9	100	n.d.		n.d.			

by a high affinity for the haemoprotein (Greim *et al.*, 1973). The larger concentrations of this detergent considerably decreased the rate of cytochrome *P*-450 reductase and completely abolished the stimulating effect of 3.2 mM-aminopyrine.

Substrate-binding capacity of cytochrome *P*-450

Another indication that 0.01% sodium deoxycholate serves as a type I substrate for cytochrome *P*-450 was its competitive inhibition of the binding of aminopyrine (Fig. 2*a*). A qualitatively different effect was detected when using sodium deoxycholate at 0.04% and higher concentrations: without changing the affinity of cytochrome *P*-450 for aminopyrine, this agent lowered the molar concentration of binding sites (constant $\Delta E_{\max.}$) for the substrate; that is, non-competitive inhibition of the binding of aminopyrine occurred. As for aniline, a type II substrate, sodium deoxycholate inhibited its binding, this being a so-called mixed inhibition, i.e. K_s for this substrate increased and $\Delta E_{\max.}$ decreased simultaneously. At the same time, increasing the sodium deoxycholate concentration also enhanced its inhibiting effect (Fig. 2*b*).

Double-reciprocal plots illustrating the influence of carbonyl cyanide *m*-chlorophenylhydrazone on aminopyrine binding prove that this agent competitively blocked substrate binding (Fig. 3*a*). However, on adding carbonyl cyanide *m*-chlorophenylhydrazone to the oxidized microsomal suspension we

failed to detect any spectra changes showing the agent binding to cytochrome *P*-450. On the other hand, Fig. 3*b*) shows that carbonyl cyanide *m*-chlorophenylhydrazone did not influence the binding of aniline, since neither the affinity for cytochrome *P*-450 nor the molar concentration of binding sites for aniline differed from the corresponding control values.

Interaction of microsomal fraction with 8-anilino-naphthalene-1-sulphonate and ethyl isocyanide

Our tests have shown a considerable fall in the quantum yield of 8-anilino-naphthalene-1-sulphonate fluorescence in the microsomal suspension after addition of carbonyl cyanide *m*-chlorophenylhydrazone or sodium deoxycholate (Fig. 4). Maximum decreases in the intensity of this fluorescence was in the presence of 60 μ M-carbonyl cyanide *m*-chlorophenylhydrazone and 0.12% sodium deoxycholate (by 60% and 87% respectively). Study of these observations by using Lineweaver-Burk plots (Fig. 5) established that sodium deoxycholate and carbonyl cyanide *m*-chlorophenylhydrazone lowered the molar concentration of the binding sites for 8-anilino-naphthalene-1-sulphonate, but did not alter the degree of affinity of this dye for microsomal phospholipids ($K_s = 5 \mu$ M). Fig. 5 also gives data on the competitive inhibition of 8-anilino-naphthalene-1-sulphonate binding to microsomal fraction by lipid-soluble anion tetraphenylborate. A rise of K_s for the dye in the presence of tetraphenylborate may

Fig. 2. Effects of sodium deoxycholate on type I (a) and type II (b) spectral changes caused by aminopyrine and aniline respectively

Incubation medium contained 0.1M-sodium potassium phosphate buffer (pH7.4). Various concentrations of aminopyrine (0.05–1.2 mM) and aniline (0.2–16.0 mM) were added to microsomal suspensions (1.6 and 3.2 mg of protein/ml respectively). From these data the K_s was calculated by using the Lineweaver–Burk plot. Assays were done in the absence (●) and in the presence of 0.01% (○), 0.04% (□) and 0.06% (■) sodium deoxycholate. Control K_s for aminopyrine was 0.2 ± 0.003 mM and that for aniline was 1.8 ± 0.09 mM. The results are means $\pm$ S.E.M. of four experiments.

apparently be explained by the displacement of 8-anilidonaphthalene-1-sulphonate by this anion from positively charged binding sites in the microsomal membrane.

Finally, comparison of ethyl isocyanide difference spectra of $\text{Na}_2\text{S}_2\text{O}_4$ -reduced haemoprotein, where the ratio of relative heights of the 455 and 430 nm peaks is a sensitive indicator of the degree of hydrophobicity of the cytochrome P-450 haem-group environment (Imai & Siekevitz, 1971), did not yield any difference between the control and carbonyl cyanide *m*-chlorophenylhydrazone-treated microsomal fraction (results not shown).

Kinetic constants of aminopyrine and aniline metabolism

Table 2 shows that increasing concentrations of sodium deoxycholate gradually inhibit aminopyrine

Fig. 3. Effects of carbonyl cyanide *m*-chlorophenylhydrazone on type I (a) and type II (b) spectral changes caused by aminopyrine and aniline respectively

Experimental details are given in legend to Fig. 2. Assays were done in absence (●) and in the presence of 20 (■) and 60 μM (□) carbonyl cyanide *m*-chlorophenylhydrazone.

metabolism, and in the presence of 0.12% sodium deoxycholate *N*-demethylation of aminopyrine practically does not occur. Since we know the V value and the content of the enzyme (cytochrome P-450), we determined the rate of dissociation of the enzyme–substrate complex (constant k_{+2}) in the control and experimental microsomal preparations. Inspection of the k_{+2} values for each sodium deoxycholate concentration indicated that this reaction was not rate-limiting during metabolism of aminopyrine. This applies to an even greater extent to aniline *p*-hydroxylation, since the k_{+2} values were greater than the V values for each sodium deoxycholate concentration.

As shown in Table 2, carbonyl cyanide *m*-chlorophenylhydrazone lowered the rate of aniline *p*-hydroxylation only slightly. Moreover, this agent did not change the content of cytochrome P-450 in the microsomal suspension after incubation at 37°C for 20 min. Hence, we infer that carbonyl cyanide *m*-chlorophenylhydrazone does not influence the kinetics of aniline metabolism in microsomal mono-oxygenases. However, this agent considerably inhibited the rate of aminopyrine *N*-demethylation; the

Fig. 4. Intensity of 8-anilidonaphthalene-1-sulphonate fluorescence in control and experimental rat liver microsomal fractions

Incubation medium contained 0.125M-KCl and 0.02M-Tris/HCl buffer (pH 7.4). Microsomal protein content in control and experimental cuvettes was the same. Cuvette volume was 2.5 ml and 8-anilidonaphthalene-1-sulphonate concentration 10 μ M. Excitation was at 360 nm and emission at 470 nm. Assays were done in control (●) and sample microsomal fraction treated with 10 (▲) and 30 μ M (△) carbonyl cyanide *m*-chlorophenylhydrazone, or with 0.03 (■) and 0.09% (□) sodium deoxycholate.

Fig. 5. Double-reciprocal plots of 8-anilidonaphthalene-1-sulphonate-microsomal fraction fluorescence against 8-anilidonaphthalene-1-sulphonate concentration

Incubation medium and experimental details are given in legend to Fig. 4. Microsomal fractions were adjusted to 1.3 mg of protein/ml. Assays were done in absence (●) and in the presence of 30 μ M-carbonyl cyanide *m*-chlorophenylhydrazone (▲), 0.03% sodium deoxycholate (■) or 30 μ M-tetraphenylborate (○). The K_s of control microsomal fraction for 8-anilidonaphthalene-1-sulphonate was 5.0 ± 0.90 M. Data represent means $\pm$ S.E.M. for six experiments.

decrease was more than 50% of the control rate, even though the content of cytochrome *P*-450 was unchanged.

A study of the pH-dependence of the inhibition of aminopyrine *N*-demethylase by carbonyl cyanide *m*-chlorophenylhydrazone showed that maximum inhibition occurred at pH 5.5–6.0, with a considerable loss of inhibition when the pH of incubation medium was made more alkaline (Fig. 6).

Discussion

The present studies on the effects of sodium deoxycholate were performed because detergents are now widely used in the study of biomembranes, both to isolate components and to probe the structure of the membrane itself (Kirkpatrick *et al.*, 1974). The conversion of cytochrome *P*-450 into cytochrome *P*-420 in the presence of sodium deoxycholate (Fig. 1*a*) may result from the effects of this detergent on hydrophobic bonds in the lipoprotein membrane (Victoria & Barber, 1969), with consequent unmasking of the hydrophobic phospholipid groups responsible for maintaining the spectral properties of the

CO-binding haemoprotein (Omura & Sato, 1964; Imai & Sato, 1967; Ichikawa & Yamano, 1967). Sodium deoxycholate 'opens' the hydrophobic cavity, created by these phospholipid groups, making it accessible to the aqueous phase (Raykhan *et al.*, 1972).

The inhibition of aniline binding and metabolism by sodium deoxycholate are not unexpected, since it is known that binding of this substrate takes place in the lipophilic moiety of the cytochrome *P*-450 molecule (Ichikawa & Yamano, 1967) and that *p*-hydroxylation of aniline by mono-oxygenase requires phospholipid (Imai & Sato, 1960; Lu *et al.*, 1972).

The results shown in Table 1 and Fig. 3(*a*) give evidence that at concentrations exceeding 0.03%, sodium deoxycholate acts as a non-competitive inhibitor of aminopyrine binding and suppresses the activity of NADPH-dependent reduction both of cytochrome *P*-450 alone and of the cytochrome *P*-450-aminopyrine complex. It is known that the 'binding protein' for type I substrates and NADPH-cytochrome *P*-450 reductase is strictly dependent for activity on the state of their surrounding phospho-

Table 2. *Effects of carbonyl cyanide *m*-chlorophenylhydrazone and sodium deoxycholate on the amount of cytochrome P-450 and kinetic constants of drug metabolism in the microsomal fraction*

Experimental conditions are given in the Material and Methods section. *V* is expressed as nmol of formaldehyde (for aminopyrine) or *p*-aminophenol (for aniline) formed/min per mg of microsomal protein. k_{+2} is expressed as *V*/nmol of cytochrome P-450 for every concentration of agents used. All the values given are the means of four to six determinations, the results of which differed by less than 5%.

Carbonyl cyanide <i>m</i> -chlorophenylhydrazone concn. (μ M)	Cytochrome P-450		Aminopyrine				Aniline			
	(nmol/mg of protein)	(% of control)	<i>V</i>		k_{+2} (min ⁻¹)		<i>V</i>		k_{+2} (min ⁻¹)	
			(% of control)		(% of control)		(% of control)		(% of control)	
0 (control)	0.96	100	3.35	100	3.48	100	0.57	100	0.6	100
10	0.96	100	2.86	85	3.0	85	0.5	88	0.52	88
20	0.96	100	2.8	84	2.92	84	0.48	84	0.5	84
30	0.96	100	2.36	70	2.46	70	0.54	95	0.56	95
60	0.96	100	2.08	62	2.17	62	0.5	88	0.52	88
90	0.96	100	1.58	47	1.65	47	0.48	84	0.5	84
Sodium deoxycholate concn. (%)										
0 (control)	1.1	100	3.35	100	3.04	100	0.57	100	0.52	100
0.01	0.96	87	3.15	94	3.28	108	0.53	93	0.55	106
0.03	0.84	76	2.72	81	3.24	106	0.48	84	0.57	110
0.06	0.69	63	1.11	33	1.6	53	0.34	60	0.49	94
0.09	0.44	40	0.61	18	1.39	46	0.21	37	0.47	90
0.12	0.25	26	0.29	9	1.16	38	0.09	16	0.36	69

Fig. 6. *Dependence of inhibition of aminopyrine N-demethylase by carbonyl cyanide *m*-chlorophenylhydrazone on pH*

Incubation media contained potassium phosphate buffers of different pH values. Carbonyl cyanide *m*-chlorophenylhydrazone (final concentration 90 μ M) was used in all experiments. The insert shows dependence of aminopyrine N-demethylase activity on pH. For experimental conditions see the Material and Methods section.

lipids and the phospholipid-dependent hydrophobic zone in the microsomal membrane (Schenkman *et al.*, 1967; Remmer, 1968; Chaplin & Mannering, 1970; Strobel *et al.*, 1970; Eling & DiAugustine, 1971; Stier & Sackmann, 1973; Schuster *et al.*, 1975). We assume that the inhibition by sodium deoxycholate, below the critical micelle concentration, is explained by the alteration of this hydrophobic zone.

To check this, an attempt has been made to characterize and compare the degree of hydrophobicity of control and sodium deoxycholate-treated microsomal membranes by using 8-anilino-naphthalene-1-sulphonate, an 'indicator' of hydrophobic interactions in the microsomal fraction (Vanderkooi & Martonosi, 1969; Eling & DiAugustine, 1971). Figs. 4 and 5 show that in the presence of 0.06% sodium deoxycholate, the quantum yield of 8-anilino-naphthalene-1-sulphonate fluorescence falls markedly and that the detergent decreases the molar concentration of the binding sites for this dye, but does not affect the affinity of 8-anilino-naphthalene-1-sulphonate for phospholipids. A similar tendency is observed for the action of 0.04–0.06% sodium deoxycholate on the binding kinetics for aminopyrine (Fig. 2a). Taking into account the assertion of Eling & DiAugustine (1971) that the 'high-affinity' sites for 8-anilino-naphthalene-1-sulphonate are the apolar hydrocarbon chains of

Scheme 1.

phosphatidylcholine and that the 'binding protein' of cytochrome *P*-450 apoenzyme is the binding site for aminopyrine (Remmer *et al.*, 1968), we consider that an increase in the hydrophilicity of the phospholipid component of the microsomal membrane determines the suppression of type I-substrate binding in the presence of detergent.

Since sodium deoxycholate is an anionic detergent, we compared its effects on binding of aniline, aminopyrine and 8-anilidonaphthalene-1-sulphonate with those of the lipid-soluble anion, tetraphenylborate, which is also an inhibitor of microsomal mono-oxygenase (Liberman *et al.*, 1974). The absence of any influence of tetraphenylborate on types I and II binding spectra (not shown) and the competitive inhibition of 8-anilidonaphthalene-1-sulphonate binding (Fig. 5) apparently shows that the negatively charged hydrophilic region of the sodium deoxycholate molecule is not associated with the effects caused by this agent.

On the strength of all the factors that determine mono-oxygenase activity in the presence of 0.03–0.09% sodium deoxycholate, we conclude that in the present case the rate-limiting factor in aniline *p*-hydroxylation is the activity of cytochrome *P*-450. On the other hand, the activity of NADPH-cytochrome *P*-450 reductase can be regarded as the rate-limiting factor in aminopyrine *N*-demethylation.

The ability of carbonyl cyanide *m*-chlorophenylhydrazone to influence the mitochondrial oxidative system and to increase the electrical conductivity of artificial and natural membranes is because of the presence of an ionizable proton in its structure (see Scheme 1) (Mitchell, 1966) and its solubility in low-polarity media (Hemker, 1962) increasing the permeability of the membranes to H^+ ions (Liberman, 1968).

The present results show that incubation of microsomal fraction in the presence of increasing amounts of carbonyl cyanide *m*-chlorophenylhydrazone is not accompanied by changes in the spectrum of cytochrome *P*-450 as well as in the binding and metabolism of aniline. This suggests that the hydrophobic region of the microsomal membrane, including the active centre of mono-oxygenase for type II substrates, is not influenced by carbonyl cyanide *m*-chlorophenylhydrazone. Indeed, the ethyl isocyanide difference spectra of cytochrome *P*-450 did not differ considerably for the control and protonophore-treated

microsomal fractions, which points to the intactness of the hydrophobic region containing the haem group of cytochrome *P*-450 (Imai & Siekevitz, 1971).

However, carbonyl cyanide *m*-chlorophenylhydrazone inhibits 8-anilidonaphthalene-1-sulphonate fluorescence, diminishing thereby the molar concentration of the binding sites, but without affecting the K_s for this dye (Figs. 4 and 5). Not changing the content of type I 'binding protein' and not being a substrate for cytochrome *P*-450, carbonyl cyanide *m*-chlorophenylhydrazone nevertheless increases the K_s for aminopyrine. Lastly, the protonophore suppresses the activity of such phospholipid-dependent reactions as NADPH-cytochrome *P*-450 reductase, and even to a greater extent, *N*-demethylation of aminopyrine.

As shown in Table 2, the rate constant, k_{+2} , refers to a rate-limiting reaction in the metabolism of aminopyrine in the presence of carbonyl cyanide *m*-chlorophenylhydrazone. The mechanism of cytochrome *P*-450 action commonly accepted includes dissociation of 'reduced *P*-450–substrate-activated oxygen' complex, subsequent incorporation of one of the oxygen atoms into the substrate and release of the hydroxylated product, water and oxidized cytochrome *P*-450 (Estabrook *et al.*, 1971).

Being a weak acid and having pK_a 5.7, carbonyl cyanide *m*-chlorophenylhydrazone is most effective as a proton carrier in the presence of a mixture of its anionic and undissociated forms. At pH values on either side of its pK_a (where either the agent is in the anionic form and there are few H^+ ions or it is largely undissociated) carbonyl cyanide *m*-chlorophenylhydrazone increasingly loses its ability to be a mobile proton carrier (Skulachev, 1969). The pH dependence of *N*-demethylase inhibition by carbonyl cyanide *m*-chlorophenylhydrazone showed the maximum effect near the pK_a for this agent (Fig. 6). On the alkaline side of the pK_a the degree of inhibition falls considerably, indicating that the protonated form is responsible for the decreased *N*-demethylase activity.

The above findings suggest that the mechanism of carbonyl cyanide *m*-chlorophenylhydrazone action consists of the electrostatic interaction of its protonated form with one of the forms of activated oxygen, i.e. it is assumed that H^+ ion competes with aminopyrine for O_2^{2-} or O_2^{2-} .

Thus there may be at least two different phospholipid-dependent hydrophobic zones coupled with

cytochrome *P*-450. One of them reveals selective sensitivity to the influence of carbonyl cyanide *m*-chlorophenylhydrazone and includes 'binding protein' for type I substrates and NADPH-cytochrome *P*-450 reductase; the other contains the cytochrome *P*-450 haem group and binding sites for type II substrates. Sodium deoxycholate is capable of influencing both zones.

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