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Bifunctional compound study of the active-centre location of cytochrome *P*-450 in a microsomal membrane ('float' molecules method)

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A new approach, which we call 'float' molecules method for the determination of the active-centre location of cytochrome *P*-450 in a microsomal membrane, is proposed. We have synthesized new bifunctional compounds with the general formula:

$$R-(CH_2)_n-S-CH_2-CH(PO_3H_2)_2, \qquad \text{(I–VI)}$$

where $n = 0,3,5,6,7,10$ and R is a naphthalene-containing radical. The compounds inhibit oxidation and binding of cytochrome *P*-450 substrates of type I (naphthalene, aminopyrine) and of type II (aniline). The inhibition is of a competitive character. Compounds (I–V) neither affect NADPH-cytochrome *c* reductase, nor induce conversion of cytochrome *P*-450. A lipid-soluble fluorescent probe (1,6-diphenyl-1,3,5-hexatriene) has been used to show that these compounds do not affect melting of microsomal membrane. The ^{31}P-NMR method has demonstrated compound (III) to be incorporated into microsomal membrane so that the hydrophilic part is in the water phase. The data obtained make it possible to estimate the distance (r) between the membranes surface and Fe^{3+} in the active centre of the enzyme ($r \lesssim 20$ Å) under the assumption that all molecules of cytochrome *P*-450 are equally remote from the membrane surface.

Introduction

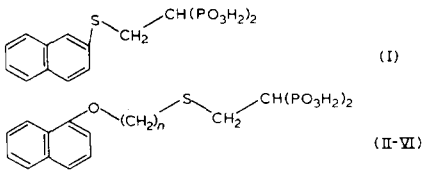
Cytochrome *P*-450 is a universal enzyme capable of oxidizing a great number of endogenous and exogenous substrates [1]. However, there are no reliable experimental data on the location of the enzyme and its active centre in a microsomal membrane. Solution of this problem is of fundamental importance for understanding the binding mechanism of various substrates with the active centre of cytochrome *P*-450, and mechanisms of catalytic acts.

The basic method to study the location of membrane-bound proteins in membranes is treatment of preparations with various proteolytic enzymes. The microsomal fraction prepared from liver of control rats was treated [2,3] with trypsin and pronase, as well as with pronase together with ultrasonic treatment. According to those experiments, the whole of cytochrome *P*-450 is in the hydrophobic zone of a microsomal membrane and is close to its inner surface. Later, Cooper et al. [4,5] used various proteinases and detergents, and demonstrated that microsomes from phenobarbital-treated rat liver contain predominantly one form of cytochrome *P*-450 with a molecular

weight of some 52 000 located in the vicinity of the outer surface of the membrane. In control microsomes, about 50% of cytochrome *P*-450 are near the outer surface, the rest being distributed between the inner surface and the hydrophobic zone of the membrane. This approach, however, provides no information on the depth of location of enzyme active centres in a microsomal membrane.

An attempt was made [6] to measure the distance between the spin-labelled metyrapone, a cytochrome *P*-450 inhibitor, located at the active centre of phenobarbital-induced microsomal cytochrome *P*-450, and a broadening agent, $Fe(CN)_6^{3-}$, distributed in the water phase. Ferricyanide proved to have no effect upon the EPR signal intensity of spin-labelled metyrapone. Therefore, Ruf and Nastainzyk [6] could only obtain the lower estimate of the distance (r) between the hydrophilic surface of the membrane and the $>N-O^{\cdot}$ group of the spin label, $r \geqslant 8$ Å.

We think that the problem of cytochrome *P*-450 active-centre location in a microsomal membrane under physiological conditions can be solved in experiments with the following compounds:



where $n = 3,5,6,7,10$. The fact that compounds (I–VI) have a polar diphosphonic acid radical and a varying aliphatic part at one end of molecules allows one to assume these molecules to be incorporated into phospholipid membranes. There is evidence [7] that trimethylalkylammonium halogenides are incorporated into egg phosphatidylcholine liposomes. If the aliphatic chain-length varies from 1 to 5–6 carbon atoms, the molecules are adsorbed on the membrane surface, whereas at a greater number of atoms they penetrate into the hydrophobic area. On the other hand, the fact that our compounds involve a naphthalene radical at the end of the aliphatic chain implies the penetration of this part of molecules into the area of the active centre of cytochrome *P*-450 since naphthalene is a substrate of type I of this enzyme and oxidizes to α-naphthol in the microsomal fraction [8].

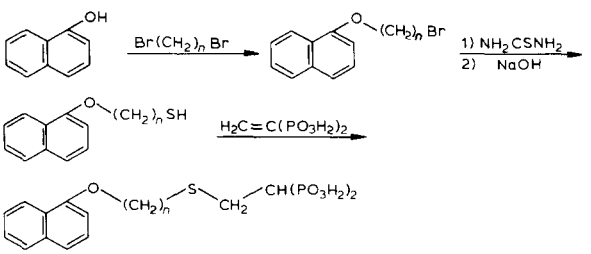
By varying the aliphatic chain-length from the polar 'head' to the naphthalene radical, we hoped to measure the depth of cytochrome *P*-450 active-centre location in a microsomal membrane by means of these 'float' molecules.

Preliminary results of this work were reported at the XVIth Conference of FEBS, Moscow, June 1984.

Materials and Methods

Chemicals. We used the reagents: catalase and glucose oxidase (p/o Biopreparat, U.S.S.R.), NADPH (Reanal, Hungary), cytochrome *c* (Serva, F.R.G.), Triton X-100 (Sigma, U.S.A.), naphthalene (Chemapol, Czechoslovakia); the other reagents were national products of either chemically pure or specially pure grade. Compound I was kindly provided by Dr. N.V. Mikhalin (Institute of Chemical Kinetics and Combustion, Novosibirsk).

Synthesis of ω-(α-naphthoxi)-3-thia-alkyliden-1,1-diphosphonic acids. Compounds (II–VI) were synthesized by the general scheme:



The synthesized compounds (II–VI) were isolated in the form of disodium salts and were identified by elemental analysis and ^1H-, ^{31}P-NMR methods *. Vinylidenediphosphonic acid was obtained as described in Ref. 9.

The disodium salt of 1-hydroxyoctylidene-1,1-diphosphonic acid, $H_3C-(CH_2)_6-COH(PO_3H_2)_2$ (VII), was obtained as described in Ref. 10.

For compounds (I–VI), the absorption bands were registered in the ultraviolet region. The parameters of typical absorption peaks are λ_{max} =

* A detailed description of the synthesis and properties of compounds (II–VI) will be published elsewhere.

215 nm, $\epsilon = 2.8 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$; $\lambda_{max} = 250$ nm, $\epsilon = 2.6 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$ for aqueous solutions of (I) and $\lambda_{max} = 216$ nm, $\epsilon = 4 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$; $\lambda_{max} = 288$ nm, $\epsilon = 8 \cdot 10^3$ $M^{-1} \cdot cm^{-1}$ for (II–VI).

Methods. Microsomes were prepared from livers of male Wistar rats (150–200 g) as described in ref. 11. The microsomal protein concentrations were measured by the method of Lowry et al. [12], the cytochrome *P*-450 concentration was determined as described by Omura and Sato [13].

The microsomal oxidation of aminopyrine, naphthalene, and aniline was tested by the product yield: formaldehyde [14], α-naphthol [8], *p*-aminophenol [15], respectively. To incorporate compounds (I–VI) into the microsomal membrane, a solution of (I–VI) in 100 mM Tris-HCl buffer (pH 7.6) was added to the microsomes, the mixture being preliminarily incubated for 5 min at 37°C. Thereafter, the substrate was introduced, and the reaction was started by NADPH. The reaction mixture contained: 100 mM Tris-HCl buffer (pH 7.6), 2.55 mg/ml microsomal protein (1.1 μM *P*-450), 1, 0.5, 0.25 mM (0.5, 0.25, 0.1 mM for aniline) substrate and 1 mM NADPH. After 15-min (aniline) and 20-min (aminopyrine, naphthalene) incubation, the reaction was terminated by adding trichloroacetic acid up to 5% concentration. After centrifugation (4°C, 6000 × *g*, 10 min), 0.5-ml samples were taken from the supernatant and analyzed.

In our experiments on substrate binding in the presence of compounds (I–VI), the latter were introduced (up to 1 mM) into microsomal suspensions: 100 mM Tris-HCl buffer (pH 7.6), 2 mg/ml microsomal protein, 1.02 μM *P*-450 in the reference and sample cuvettes of a spectrophotometer in the same buffer and, prior to measurements, were incubated with the microsomes for 5 min at 37°C.

The method of equilibrium dialysis was employed to determine the coefficient *K* of (I–VI) distribution between the aqueous and lipid phase. 0.2 ml of solutions of compounds (I–V) (10^{-3} M) were dialysed against 0.2 ml of a microsomal fraction (5 mg/ml of microsomal protein; solvent – 100 mM Tris-HCl buffer (pH 7.6)). Dialysis lasted for 10 h at 37°C and then (I–VI) concentrations in the aqueous phase were measured spectrophotometrically. By taking into account the lipid phase volume *, (I–VI) concentrations in the lipid phase were estimated and finally *K* was determined as follows: *K* = concentration in lipid phase/concentration in aqueous phase.

Our experiments on melting the microsomes with and without incorporated compounds (I–VI) were performed with the microsome preparations diluted after preliminary incubation (see above, final concentration of 0.5 mg/ml protein). A fluorescent probe 1,6-diphenyl-1,3,5-hexatriene in tetrahydrofurane was introduced (up to $4 \cdot 10^{-7}$ M) directly before measurement.

The activity of NADPH-cytochrome *c* reductase was determined spectrophotometrically from variations in the absorption peak of reduced cytochrome *c* ($\lambda_{max} = 550$ nm, $\epsilon = 2.1 \cdot 10^4$ $M^{-1} \cdot cm^{-1}$) [16]. The effect of (I–VI) upon cytochrome *P*-450 concentration was examined in two ways: (i) reduction by sodium dithionite, and (ii) reduction by NADPH under anaerobic conditions (100 mM Tris-HCl buffer (pH 7.6), 1–2 mg/ml microsomal protein, 100 mM glucose, 0.2 mg/ml glucose oxidase, 0.01 mg/ml catalase and 1 mM NADPH).

The spectrometric measurements were made on a Beckman DB-GD spectrophotometer. The difference spectra were taken on a Hitachi-556 spectrophotometer. The fluorescence depolarization was measured on a setup described by Grusdev et al. [17]. The ^1H- and ^{31}P-NMR spectra were taken on a Varian-XL-200 spectrometer **.

Experiments on quasi-elastic light scattering [23] (scattering angle 90°; wavelength of helium-neon laser 6328 Å) were carried out using a system with a multichannel digital correlator. A contribution of large contaminating species ($R \approx 5000$–$10\,000$ Å; dust, for example) was taken into account in the calculations. The samples (1 mg/ml of microsomal protein, 3 mM of (I–V) in 100 mM Tris-HCl buffer (pH 7.6)) were preliminarily incubated for 5 min at 37°C.

* The volume of the lipid phase was determined at a protein-to-lipid ratio in microsomal fraction equal to 0.6:0.4 (see, for example, Ref. 2). We assumed one lipid molecule was approx. 1200 $Å^3$ [22] and the lipid molecular weight was approx. 750.

** All solutions in NMR experiments were made in 100 mM Tris-HCl buffer (pH 7.6).

Results

Fig. 1 demonstrates changes typical for (I–VI) differential spectra of binding with a microsomal fraction. The values of $\lambda_{min} = 420–425$ nm and $\lambda_{max} = 385–390$ nm are usually observed for binding of substrates of type I [18]. Table I lists maximum amplitudes of spectral changes, and spectral dissociation constants determined for (I–VI). The influence of an aliphatic chain upon the spectral changes was examined using compound (VII). It turned out that (VII) did not give binding spectra with microsomal cytochrome *P*-450. This fact suggested that the spectral changes observed in the case of (I–VI) were induced by the interaction of cytochrome *P*-450 with the aromatic component of these molecules rather than their aliphatic chain.

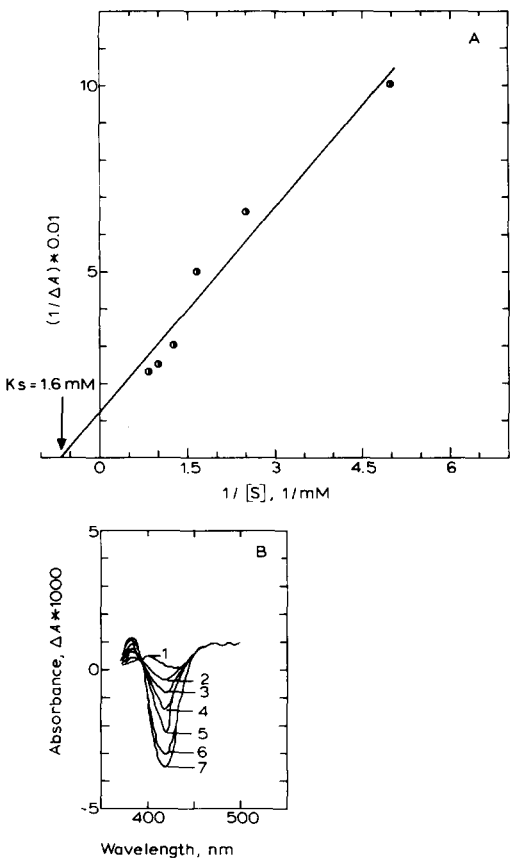


Fig. 1. Interaction of compound (III) with liver microsomal cytochrome *P*-450 (A). Microsomes were diluted in 100 mM Tris-HCl (pH 7.6) buffer up to 1.75 mg/ml protein, [*P*-450] = 0.75 μM and the magnitude of absorbance change (ΔA) was determined after successive additions of a solution of compound (III) in the same buffer (B); the concentrations of compound (III) were 0; 0.2; 0.4; 0.6; 0.8; 1; 1.2 mM for lines from 1 to 7. The difference spectra were recorded at 31°C.

We examined the effect of (I–VI) on the reactivity of microsomal carriers, and also the ability of (I–VI) to convert cytochrome *P*-450 into the enzymatically inert form *P*-420. Compounds (I–V) did not suppress NADPH-cytochrome *c* reductase activity up to a concentration of 3 mM. At the same time, (VI) inhibited this activity by 80% even at 0.4 mM in the same conditions. The latter fact led us to conclude that compound (VI) was not suitable for solving our problem.

With sodium dithionite as a reductant, compounds (III–V) proved to induce 20–50% conversion of *P*-450 into *P*-420 at concentrations of 1–3 mM. However, in the presence of (I–V) in the microsomal system, no conversion of *P*-450 occurred with NADPH as a reductant. The concentrations and incubation times used in this case corresponded to conditions of microsomal oxidation (see Materials and Methods).

We investigated also the (I–V) effect on the state of the phospholipid component of a microsomal membrane by the method of depolarization of diphenylhexatriene probe fluorescence. Compounds (I–V) did not affect the curves of melting of microsomes measured by this method.

Compounds (I–V) inhibited microsomal oxidation of aminopyrine, naphthalene, and aniline. As seen from Fig. 2 (A,B), (I–V) inhibit oxidation of substrates of type I, aminopyrine and naphthalene, the inhibition efficiency increasing with the aliphatic chain-length in the series I–V. At the same time, (I) and (II) do not inhibit oxidation of aniline (substrate of type II, see Fig. 2C); at low concentrations they even slightly increase the *p*-aminophenol yield. The *p*-aminophenol yield is inhibited for (III–V) only, i.e., starting with $n = 5$. In order to study the influence of amphiphilic compounds on the microsomal system, one has to know the coefficient of their distribution between the aqueous and lipid phases. The distribution coefficient K obtained by equilibrium dialysis (see Materials and Methods) was found to be independent of the aliphatic chain-length, n. They are: $K = (7.0 \pm 0.1) \cdot 10^2$ for compound (I) and (4.2 $\pm$

TABLE I

MAXIMUM AMPLITUDES OF SPECTRAL CHANGES (ΔA_{max}) AND SPECTRAL DISSOCIATION CONSTANTS (K_s) FOR COMPOUNDS (I–VI)

Microsomal suspension contained 100 mM Tris-HCl (pH 7.6), 1.75 mg/ml microsomal protein, 0.75 μM *P*-450 and compounds (I–VI) were added to the same buffer. Temperature 31°C. Note that compound (I) has a chemical structure different from that compounds (II–VI). The characteristics of the ultraviolet sectra and the coefficient K of distribution between the aqueous and lipid phases for compound (I) differ also from those for (II–VI) (see text). Therefore, characteristics for compound (I) are not obviously in the same series as those for (II–VI).

Compound:	I	II	III	IV	V	VI
ΔA_{max} (absorbance)	0.0023	0.0013	0.0041	0.0059	0.0067	0.011
K_s (mM)	2.7	2	1.6	1	0.64	0.175

$0.15) \cdot 10^2$ for compounds (II–VI). Thus, the observed increase in the microsomal oxidation inhibition by compounds (II–V) with growing chain-length n (Fig. 2) cannot be explained by the increase of the inhibitor concentration in the lipid phase.

Experiments on quasi-elastic light scattering have provided an additional evidence for the absence of the effect of compounds (I–V) on the microsomal membrane. The compounds (I–V) affected neither the amount nor the size of microsomal species ($R \approx 1000$ Å). This observation indicates that the compounds do not produce mixed micelles with microsomal lipids, i.e., solubilization of microsomes does not occur, formation of smaller structures ($R \approx 100$ Å) from microsomal species

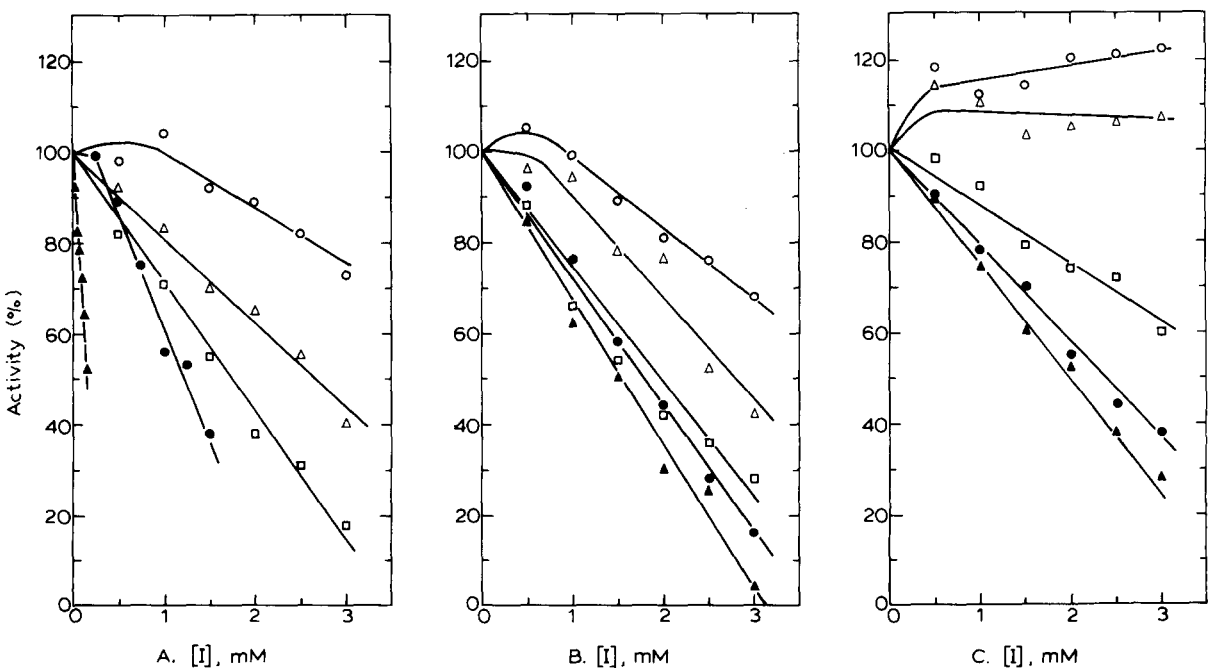


Fig. 2. Inhibition of microsomal oxidation of substrates of cytochrome *P*-450 by compounds: I (○), II (△), III (□), IV (●), V (▲). The substrates are: aminopyrine (A), naphthalene (B), aniline (C). The initial concentrations of the substrates are 1, 1, 0.5 mM, respectively. The incubation mixture contained 100 mM Tris-HCl buffer (pH 7.6), 2.5 mg/ml microsomal protein, [*P*-450] = 1.1 μM, [NADPH] = 1 mM.

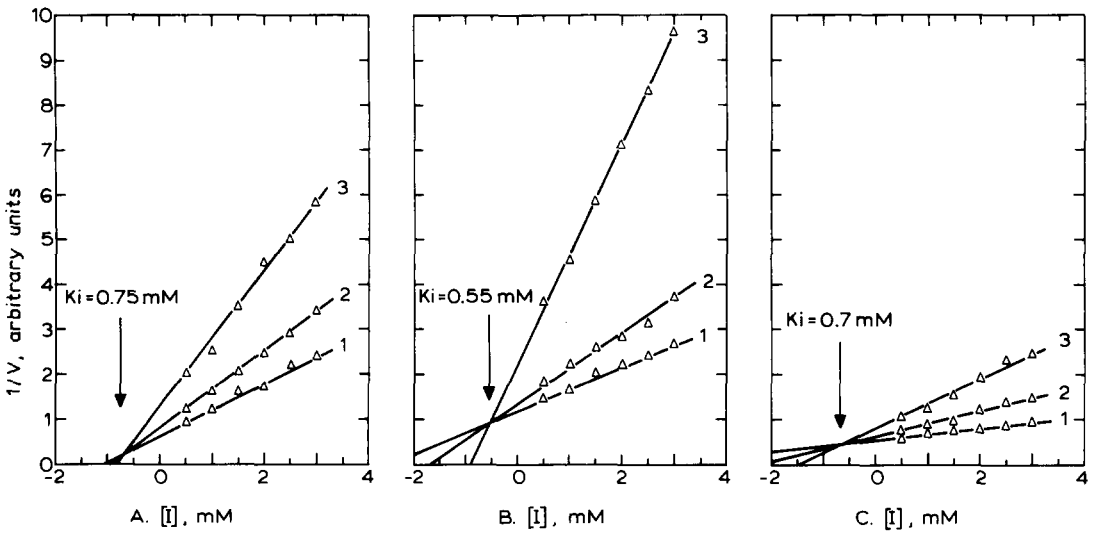


Fig. 3. The Dixon-plots of oxidation inhibition of aminopyrine (A), naphthalene (B) and aniline (C). [I], concentration of compound (III). Concentrations of substrates are (for A, B, C correspondingly) (1) 1, 1, 0.5 mM; (2) 0.5, 0.5, 0.25 mM; (3) 0.25, 0.25, 0.1 mM. The content of the other components is as in Fig. 2. $1/V$, an arbitrary unit is 1.5, 1.2, and 9.4 min/μmol per mg microsomal protein for A, B and C, respectively.

TABLE II

INHIBITION CONSTANTS (K_i) FOR COMPOUNDS (I–V) IN MICROSOMAL OXIDATION OF VARIOUS SUBSTRATES

Inhibition conditions are described in Materials and Methods.

K_i (mM)	Aminopyrine	Naphthalene	Aniline
I	6.6	2.8	–
II	2.25	1.9	–
III	0.75	0.55	0.7
IV	0.32	0.38	0.67
V	0.034	0.15	0.6

occurred by addition up to 3% (v:v) of Triton X-100.

As a typical example, see in Fig. 3 the experimental data on the oxidation inhibition of aminopyrine, naphthalene and aniline of compound (III). Similar experiments were performed with the use of (I–V). In all the cases, the inhibition (if any) was of a competitive character. The inhibition constants are listed in Table II.

We examined the (I–V) effect on binding of aminopyrine and aniline with microsomal cytochrome *P*-450 (see Table III). In the case of aminopyrine, the amplitude of spectral changes reduces gradually as inhibitor chains grow (no spectral changes in the presence of (V)). At the same time, the influence of compounds (II–V) on aniline binding is less pronounced than on aminopyrine binding, the former being significant at $n = 7$.

Although there are data [19] on the possibility

TABLE III

MAXIMUM AMPLITUDES OF SPECTRAL CHANGES (ΔA_{max}, ABSORBANCE) IN BINDING SPECTRA OF ANILINE AND AMINOPYRINE IN THE PRESENCE OF COMPOUNDS (I–V)

For experimental conditions see Materials and Methods. Also, see legend to Table I for note about compound (I).

	Control	I	II	III	IV	V
Aniline	0.0125	0.01	0.013	0.01	0.0095	0.006
Aminopyrine	0.0072	0.007	0.0055	0.0035	0.0025	–

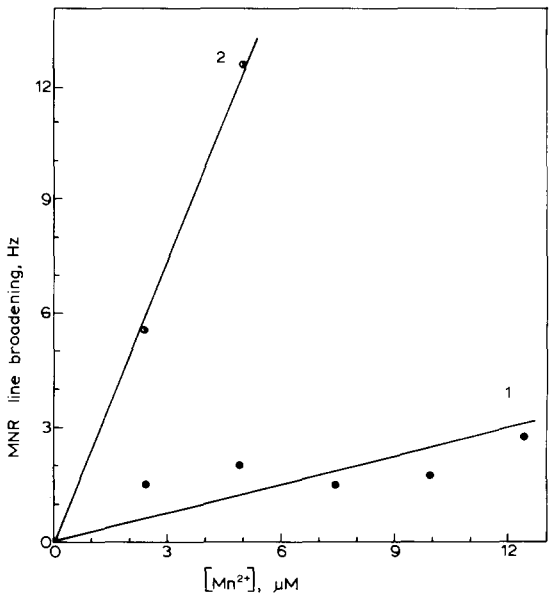


Fig. 4. Paramagnetic broadening of ^{31}P-NMR signal of compound (III) (10^{-2} M). (1) In the presence of a microsomal fraction (10 mg/ml of microsomal protein); (2) without microsomes. Buffer: 100 mM Tris-HCl (pH 7.6). $T = 22°C$.

of incorporating compounds with a polar 'head' and a nonpolar 'tail' into membranes, it is necessary to determine whether compounds (I–V) are incorporated into a membrane so that the hydrophilic 'head' is in the surface hydrophilic layer rather than penetrates into the hydrophobic zone. For that purpose we checked the phosphate groups of (I–V) using compound (III) for complexing with Mn^{2+} which are exclusively in the water phase. The paramagnetic broadening $\Delta\nu$ obeyed the formula [20]:

$$\pi \cdot \Delta\nu = \frac{q \cdot [MnL]}{[L]} \cdot \frac{1}{T_2 + \tau}$$

where [MnL] is the concentration of the complex, [L] is the free ligand concentration, q is the coordination number of a paramagnetic ion, T_2 is the nuclear transverse relaxation time in the complex, τ is the ligand lifetime in the complex. Fig. 4 shows linear dependences of $[Mn^{2+}]$ on ^{31}P signal broadening of compound (III) in the presence and absence of microsomes. The broadening is less pronounced in the presence of microsomes, which can be due to decreasing molar fraction of the complex MnL (see the above equation) because the microsomal fraction has other sites (phosphate groups of microsomal lipids, ribosomes, etc.) capable of forming complexes with Mn^{2+}.

Of principal interest is the type of exchange between Mn^{2+} and phosphate groups of low-molecular-weight ligands. As can be seen in Table IV, the temperature rise in the presence of Mn^{2+} leads to broadening of the ^{31}P signal of compound (III) both with and without the microsomal fraction. This observation indicates that the slow exchange has been accomplished [21], i.e., $\tau > T_2$.

The ability of compound (III) to make complexes with Mn^{2+} in the presence of microsomes and the same character of exchange allow us to conclude that the hydrophilic part of (I–V) does not penetrate into the interior of a microsomal membrane *.

TABLE IV

LINEWIDTHS (Hz) OF NMR SPECTRA OF COMPOUND (III)

Note: ^{31}P-NMR signals of compounds (I–VI) are similar; they are singlets at -18 ppm from free H_3PO_4.

Condition	Linewidth (Hz)	
	Without microsomes	In the presence of microsomes (10 mg/ml of microsomal protein)
10^{-2} M, 22°C	6.9	8.4
+2.5 μM $MnCl_2$, 22°C	12.4	10.0
+2.5 μM $MnCl_2$, 37°C	16.5	12.4

* For the reaction $Mn^{2+} + L \overset{K}{\rightleftarrows} MnL$, $[L]_0 \gg [Mn^{2+}]_0$ under the experimental condition employed. Hence, at $K \cdot [L]_0 \gg 1$ (stability constants K of these complexes $\approx 10^7$ M^{-1} [24]), the concentration of the complex ($[MnL] = K \cdot [Mn^{2+}]_0[L]_0/(1 + K \cdot [L]_0)$) is independent of temperature and is equal to $\approx [Mn^{2+}]_0$. In addition, the linewidths (≈ 1 Hz) of ^{1}H-NMR spectra of (I–V) did not change upon variation of their concentrations from $5 \cdot 10^{-4}$ to 10^{-2} M, neither did they depend on temperature variations from 13 to 37°C. This fact indicates that the critical micelle concentration for (I–V) is higher than 10^{-2} M. Consequently, the $q[MnL]/[L]$ ratio (see equation in text) remains unchanged under the experimental conditions. The observed changes in the linewidths with the temperature (see Table IV) are due to the variations of τ exclusively.

Discussion

Our experiments suggest that compounds (I–V) neither inhibit NADPH-cytochrome *c* reductase activity, nor induce conversion of *P*-450 into *P*-420.

Our experiments based on the methods of fluorescence depolarization, quasi-elastic light scattering and ^{31}P-NMR have demonstrated that compounds (I–V) do not induce dramatic changes in the structure of a microsomal membrane and are incorporated into the latter so that the polar 'head' is in the hydrophilic region rather than in the interior of the membrane.

Our experiments on (I–V) distribution between the aqueous and lipid phases carried out by the equilibrium dialysis method have shown the distribution coefficient to be independent of n. This allows one to employ (I–V) as 'molecular scales' to estimate the distance between the hydrophilic surface of a membrane and the sites of binding the substrates of types I and II with cytochrome *P*-450, i.e., the distance to its active centres.

Compounds (I–V) are bound to microsomal cytochrome *P*-450 as type I substrates. In this case, an increase in the aliphatic chain-length results in an increase of ΔA_{max} (Table I, (II–V)) and a decrease of K_s (Table I, (I–V)) which indicates a more effective binding.

Compounds inhibit oxidation of types I and II substrates, the inhibition being of a competitive character (Fig. 3). Our data on (I–V) binding with microsomal cytochrome *P*-450 and on inhibition of microsomal oxidation of various substrates have shown that most probably the aromatic part of these compounds can penetrate into the region of active centres of cytochrome *P*-450. Note that K_i decreases with increasing the aliphatic chain-length (Table II) for type I substrates (aminopyrine, naphthalene) and is practically constant at $n =$ 5,6,7 for type II substrate (aniline).

The data on the effect of (I–V) upon the maximum amplitudes of spectral changes ΔA_{max} correspond qualitatively to the data on microsomal oxidation inhibition, i.e., ΔA_{max} decreases gradually as the chain-length grows (Table III) for type I substrates (aminopyrine), while for type II substrates (aniline) ΔA_{max} changes slightly starting with $n = 3$, notable changes being observed only for $n = 7$.

TABLE V

DISTANCES BETWEEN HYDROPHILIC GROUPS AND AROMATIC RINGS FOR COMPOUNDS (I–V) IN THE MAXIMAL-LENGTH CONFORMATION (L_{max})

For values of angles and lengths of bonds see, e.g., in The Chemist's Companion by A.J. Gordon and R.A. Ford (J. Wiley & Sons, 1972) 2. III and 2.V.B.

Compound:	I	II	III	IV	V
L_{max} (Å)	11.2	15.1	17.6	18.8	20.1

According to Ref. 18, substrates of type I are bound in the vicinity of the haem group of *P*-450, while those of type II can bind directly with Fe^{3+} of the haem. Based on this idea and using our data and the distance between the hydrophilic group and the aromatic part of our probes (Table V), it is possible to propose a model of location of microsomal *P*-450 active centres with respect to the membrane surface.

If we proceed from the simples assumption that a microsomal membrane has cytochromes *P*-450 of the same type which are distributed at the same depth, the model of location of substrate-binding zones will be as follows (Fig. 5). The hydrophobic zone of binding type I substrates is rather large and located at a depth of ≈ 11 to ≈ 21 Å from the hydrophilic surface. The zone of binding type II substrates (coordination zone near Fe^{3+}) is

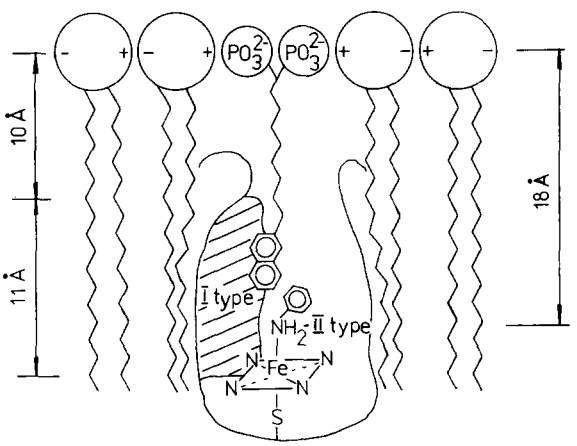


Fig. 5. A proposed model of cytochrome *P*-450 active-centre location on the assumption that all molecules of membrane-bound enzyme are equally remote from the membrane-bound enzyme are equally remote from the membrane surface.

18–23 Å under the hydrophilic surface. These estimates corresponded to the maximum distances since they were made under assumption of maximal elongation of the hydrocarbon chain (Fig. 5).

If we assume the hydrophobic layer of a membrane to have various active centres of cytochrome *P*-450 with a depth distribution, the situation will be as follows: the forms of cytochrome *P*-450, on which mainly type I substrates are oxidized, are closer to the surface. At a depth of 18 Å, the forms characterized by oxidation of type II substrates are located. The true model can be chosen by means of probes which are similar to ours but have type II rather than type I substrates at the end. Because in this work control microsomes (mixture of different isozymes) were used, we obtained mean distances. A more defined quantitative information can be obtained using induced microsomes and reconstructed systems.

We also hope this method, which can be called 'float' molecules method, will be applicable to solving the problems of location of active centres of other membrane-bound proteins.

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