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Interaction of hexane phosphonic acid diethyl ester with phospholipids in hepatic microsomes and reconstituted liposomes as studied by ^{31}P-NMR

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By use of ^{31}P-NMR, quasi-elastic light scattering and freeze-fracture electron microscopy it is shown that hexane phosphonic acid diethyl ester (PAE) is incorporated in hepatic microsomes without any alteration of the bilayer structure at two different sites. These findings proved that PAE can be used as molecular ^{31}P-NMR probe in microsomes to get information about lipid-protein interactions. Extensive studies on reconstituted liposomal systems which contained cytochrome *P*-450 and cytochrome *P*-450 reductase showed that both proteins influence the localization of incorporated PAE. The results indicate a specific interaction of phosphatidylethanolamine (PE) with cytochrome *P*-450 in microsomes.

Introduction

The interaction of liver cytochrome *P*-450, the main protein constituent of the hepatic endoplasmic reticulum with lipids (particularly PE) resulting in the concept of boundary lipids is the subject of controversial discussions about their existence in natural membranes (reviewed in Ref. 1 and references therein). With regard to *P*-450 this interaction was recently studied by fluorescence methods [2,3], by electron paramagnetic resonance [4,5] and by ^{31}P-NMR [6]. However, the existence of a boundary lipid as postulated on the basis of EPR measurements could not be proved by NMR.

^{31}P-NMR is well established in studying membrane structures [7]. However, ^{31}P-NMR of lipids cannot give reliable information about protein-lipid interactions because the amount of lipids interacting with proteins in natural membranes is low and the ^{31}P-NMR signals are broad and complex due to the large chemical shift anisotropies of the different lipids and to various dynamical processes (e.g. exchange and immobilization). By use of PAE as a phosphorus probe molecule and its incorporation in natural membranes part of these difficulties could be circumvented. PAE is a small hydrophobic molecule the ^{31}P-NMR signals of which do not interfere with those of the lipids. Therefore by ^{31}P-NMR information about the probe molecule and the lipids can be obtained simultaneously. The behaviour of PAE in artificial membranes and the physical properties of the PAE-treated membranes (e.g. structure, ion permeation) have been extensively investigated by

Abbreviations: PAE, hexane phosphonic acid diethyl ester, *P*-450, cytochrome *P*-450; *P*-450 reductase, NADPH-dependent cytochrome *P*-450 reductase, PC, phosphatidylcholine, PE, phosphatidylethanolamine; PI, phosphatidylinositol, PS, phosphatidylserine; PA, phosphatidic acid

^{31}P-NMR and other methods [8,9]. The influence of some particular phosphonic acid ester on biological membranes and whole cells poses other interesting problems [10].

Based on well established ^{31}P-NMR data of microsomes [6,11–13] the aim of this paper is to demonstrate the applicability of PAE to the analysis of lipid-protein interaction in microsomes by ^{31}P-NMR. By use of this probe experimental support is given to the assumption that PE interacts specifically with *P*-450 which represents an integral membrane protein.

Materials and Methods

Rabbit liver microsomes were prepared from phenobarbital-induced rabbits as described previously [14]. The final pellet was washed with 100 mM pyrophosphate buffer (pH 7.4) to remove ribosomes and after that two times with 50 mM Hepes buffer (pH 7.3) which contained 1 mM EDTA (buffer A) to remove pyrophosphate and detached ribosomes. The final pellet was suspended in buffer A which contained 10% ^2H_2O to a final concentration of 25–30 mg microsomal protein per ml (140–170 μM *P*-450). The protein content and the *P*-450 content were determined according to Refs. 15 and 16, respectively. By determination of the phosphorus content of the microsomes used [37] a protein-lipid ratio of 1.1 (w/w) in untreated microsomes was deduced.

Part of the pyrophosphate washed microsomes was treated with trypsin as described elsewhere [11]. The trypsinization caused a loss of microsomal proteins of about 20% whereas the *P*-450 concentration and phospholipid composition remained unchanged [11]. No changes of the pH value of the microsomal suspension were measured during the treatment. Trypsinized microsomes were also two times washed and the pellet resuspended analogously to the control.

Total microsomal lipids were extracted from microsomes according to Ref. 17 and stored at −20°C in a 1:1 chloroform/methanol mixture. The lipids were dried from this solution under vacuum and the lipid film was dispersed in buffer A containing 10% ^2H_2O at a concentration of 30 mg lipids per ml. The phospholipid composition was determined by ^{31}P-NMR according to Ref. 11: 54% PC, 27% PE, 10% PI, 9% PS. Vesicles of total microsomal lipids and of PC were prepared by ultrasonication of the final lipid dispersion in a water bath sonifier.

For reconstitution the protein components *P*-450 LM_2 and *P*-450 reductase were prepared according to Refs. 18 and 19. The reconstitution of the monooxygenatic system was performed with the cholate gel filtration method at a molar stoichiometry of *P*-450 LM_2/reductase/phospholipid = 1:0.1:600 in 100 mM Tris buffer (pH 7.4). At a cholate/phospholipid ratio = 10:1 an almost complete incorporation of all components was achieved. More than 90% of the protein components was incorporated. The formation of liposomes was morphologically controlled by electron microscopy and quasi-elastic light scattering measurements. The reductase in the liposomes was active and no cytochrome *P*-420 formation was observed. The *N*-dimethylase activity according to Nash [20] was controlled in similarly reconstituted liposomes (stoichiometry 1:0.2:600 in 100 mM phosphate buffer (pH 7.4), 2 mM EDTA and 20% glycerol). After the preparation the liposomes were concentrated by ultrafiltration (PM 30 membrane at 3 atm) to about 1 ml of 15 μM *P*-450.

^{31}P-NMR measurements were performed at 81 MHz under conditions of broad band inverse gated proton decoupling using a Bruker WP 200 spectrometer. Unless indicated otherwise, 1000 transients were accumulated using 90° radiofrequency pulses with a 2-s interpulse time. A 50 Hz line broadening was applied to the free induction de-

TABLE I

APPARENT DIAMETER (d_{app}) AND POLYDISPERSITY PARAMETER (δ_z) OF MICROSOMES AND RECONSTITUTED LIPOSOMES BEFORE AND AFTER PAE ADDITION AT 37°C AS DETERMINED BY QUASI-ELASTIC LIGHT SCATTERING

	PAE/total protein (w/w)	d_{app} (nm)	δ_z
Microsomes	0	209	0 12
	1 30	220	0 14
Trypsinized microsomes	0	187	0.11
	1 30	195	0.12
Reconstituted liposomes	0	95	0.09
	1·8	115	0.12

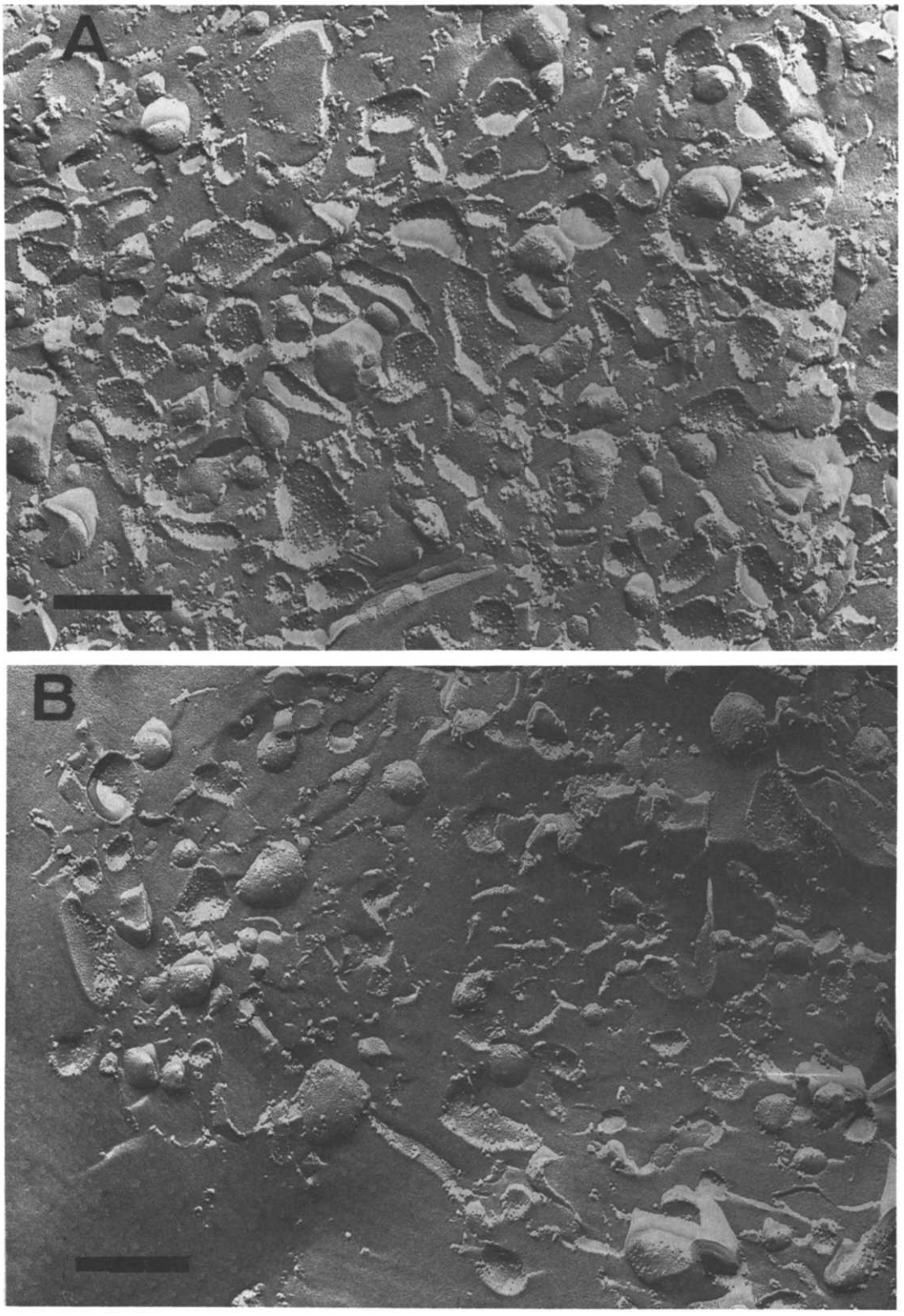

Fig 1 Freeze-fracture replicas of microsomes before (A) and after addition of PAE (B) at a ratio of phospholipid/PAE = 27 5 (w/w) The bar represents 300 nm.

cays with exception of the spectra in Fig. 10. The temperature maintenance was better than ± 1 K.

The ^{31}P-NMR chemical shift of PAE in different organic solvents and in water was measured by use of an external standard in a cylindrical capillary. The susceptibility correction was performed for each solvent.

Quasi-elastic light scattering measurements were carried out in the homodyne autocorrelation mode using a He-Ne laser (Carl-Zeiss-Jena, G.D.R.) and a 100 channel autocorrelator coupled with a computer (EMG Budapest, Hungary) as described in Ref. 11. The influence of PAE on the size of microsomes and liposomes was analyzed by quasi-elastic light scattering measurements. Table I demonstrates that PAE caused a swelling of all systems. The δ_z values which were only slightly changed as compared with untreated microsomes and liposomes indicate that no lysis of the membranes or fusion occurred at this PAE concentrations. However, one must take into account that the quasi-elastic light scattering results are z-averaged, therefore the obtained values of d_{app} (apparent diameter) may be larger than number averaged diameters measured for example by electron microscopy.

An analysis of the reduction reaction of microsomal P-450 in the presence of PAE concentrations as used in the NMR measurements revealed no significant differences in rate constant and in phase distribution as compared to the control.

The influence of PAE treatment on microsomes was studied also by freeze-fracture electron microscopy. The samples were quenched from 25°C after addition of 30% glycerol as cryoprotectant using a Balzers QFD-101 cryo jet and after that prepared in a Balzers freeze-fracture device. As can be inferred from Fig. 1 this method did not reveal any changes in the membrane structure due to PAE treatment.

PAE of highest purity was synthesized in the laboratory of Professor Grossmann, Dresden. The partition coefficient of PAE in water/n-heptane was determined to be $c_1/c_2 = 9.1 \cdot 10^{-3}$ at 25°C. PAE was added to the sample under stirring before the measurements.

Hepes and Tris buffer as well as trypsin were obtained from Serva (F.R.G.). The water used was bidistilled and ion-exchanged. All other reagents were of analytical grade and used without further purification.

Results

Fig. 2 shows ^{31}P-NMR spectra of pyrophosphate washed microsomes with sufficiently high PAE concentrations (molar ratio of microsomal lipids/PAE = 3.6) at various temperatures. At low temperatures (Fig. 2A) PAE-treated microsomes exhibit a typical bilayer lineshape which does not differ from that of control microsomes. PAE gives rise to a narrow single isotropic line at -37.7 ppm denoted as signal A. With increasing temperature (above 15°C) the microsomal lineshape changes to a narrow and more symmetrical one due to the lateral diffusion of the phospholipids [11] and a second isotropic PAE signal appears at -36.0 ppm denoted as signal B (Fig. 2B–D). The intensity ratio of the PAE signals is shifted in favour of signal B with increasing temperature up to 35°C. A further increase of temperature (up to 42°C) did not affect this ratio. All these changes were found

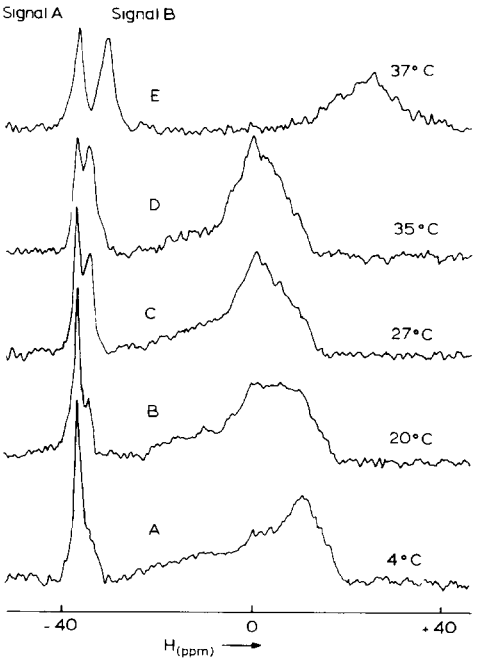

Fig. 2 81 MHz ^{31}P-NMR spectra of microsomes with PAE (phospholipid/PAE = 27 5 w/w) at the temperatures indicated (A–D) and on addition of 5 mM $EuCl_3$ (E)

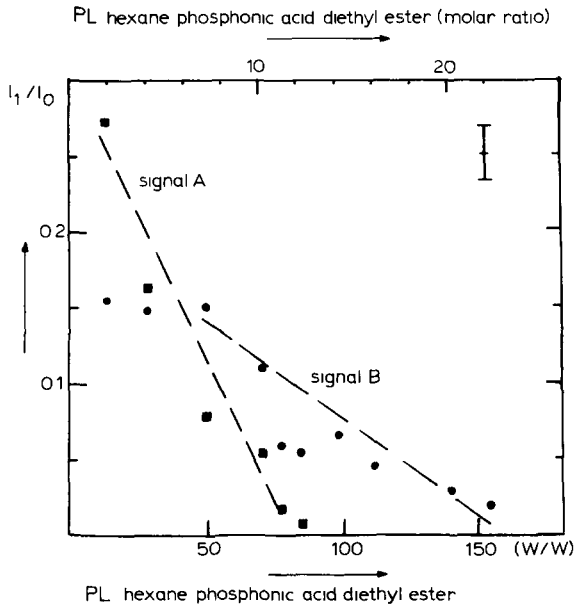

Fig 3 Intensity of signal A (■) and signal B (●), respectively, to that of the phospholipid signal (I_1/I_0) versus the PAE concentration at 37°C; 2000 scans were accumulated for each pair of data points.

to be completely reversible.

The addition of low concentrations of paramagnetic Eu^{3+} ions at 37°C caused a strong upfield shift of signal B whereas signal A remained unchanged up to concentrations of $EuCl_3$ of 30 mM (Fig. 2E). Signal B reached its maximum integral intensity at 35°C which corresponds to about 20% of that of the phospholipid. The ratio of the computer integrated absolute intensities of the PAE signals to that of the phospholipids is 0.32 at 4°C and 0.34 at 35°C. The sum over all intensities of each spectrum was not affected by the temperature.

At low PAE concentrations (below a molar ratio of microsomal lipids/PAE = 14) at 37°C only signal B was observed. With increasing PAE concentration signal A appears the intensity of which increases faster than that of signal B (Fig. 3). Above a molar ratio of microsomal lipids/PAE = 8 the intensity of signal B remained unchanged. Presumably the saturation point of site B which gives signal B is reached at this concentration. The repeated addition of PAE above this concentration increased the intensity of signal A only.

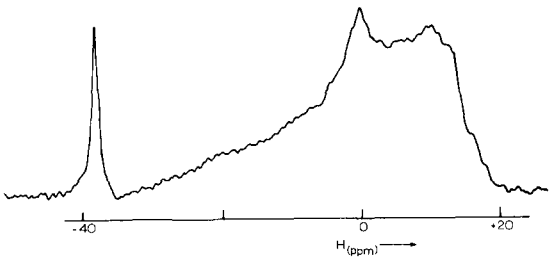

Fig. 4. 81 MHz ^{31}P-NMR spectrum of an aqueous dispersion of microsomal lipids with PAE (microsomal lipid/PAE = 20 w/w) at 37°C.

An aqueous dispersion of microsomal lipids with incorporated PAE exhibited only one signal at −37.6 ppm over the 4–37°C temperature range (Fig. 4). Contrary to signal B this signal does not shift on addition of $EuCl_3$ up to concentrations of 20 mM. These characteristics indicate that this signal corresponds to signal A in microsomes. In trypsinized microsomes (Fig. 5) and in liposomes of microsomal lipids (Fig. 6A) only signal A was observed in the 4–40°C temperature range. This signal is insensitive to temperature changes and to the addition of $EuCl_3$ up to 30 mM.

PAE dispersed as macroscopic oil droplets in water forms a signal at −33.5 ppm insensitive to

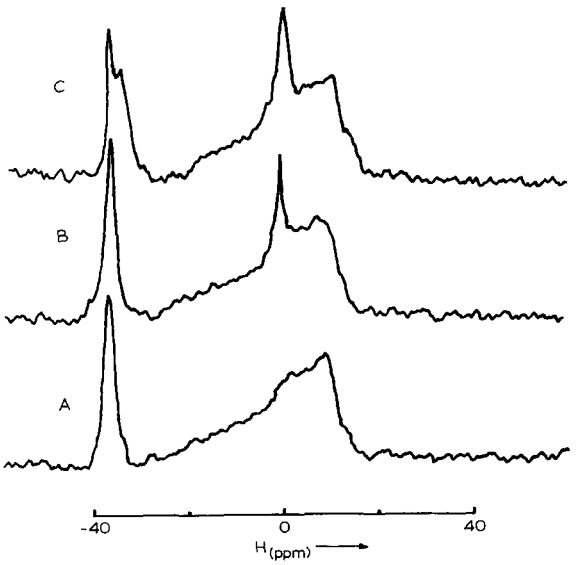

Fig. 5. 81 MHz ^{31}P-NMR spectra of trypsinized microsomes with PAE (phospholipid/PAE = 35 w/w) at 4°C (A), 37°C (B) and on addition of cytochrome *c* (total protein/cytochrome *c* = 5 w/w) at 37°C (C).

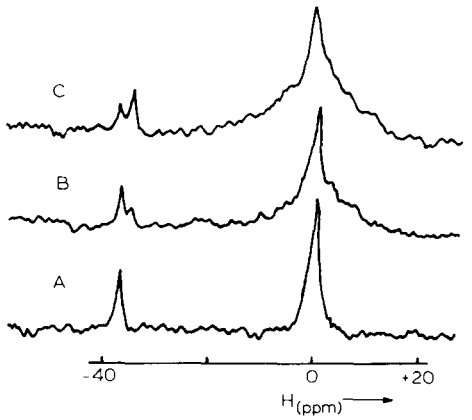

Fig. 6 81 MHz ^{31}P-NMR spectra of liposomes of microsomal lipids with PAE at 37°C (A) and on addition of pure microsomes at 37°C giving a ratio of total protein/PAE in the mixture of 10 (B) and of 25 (C) by wt., 2000 scans were accumulated for each spectrum

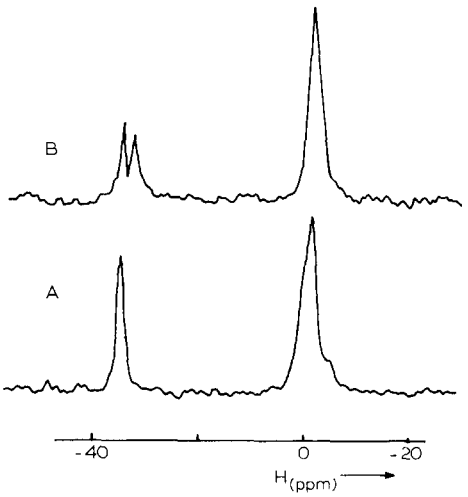

Fig 7 81 MHz ^{31}P-NMR spectra of *P*-450 reconstituted in liposomes of microsomal lipids with PAE (protein/PAE = 8 w/w) at 37°C (A) and of *P*-450 and *P*-450 reductase reconstituted in microsomal lipids with PAE under identical conditions (B) 2500 scans were accumulated for each spectrum

temperature changes and the addition of shift reagents (see below). This evidences that neither signal A nor signal B results from PAE droplets in water. The chemical shift of PAE in organic solvents was found to be strongly dependent on the solvent (Table II).

The addition of cytochrome *c* to trypsinized microsomes with PAE at 37°C produces signal B with the same characteristics as reported above (Fig. 5C). The addition of cytochrome *c* to an aqueous dispersion or liposomes of microsomal lipids with incorporated PAE, however, did not have any effect.

The addition of pure microsomes to liposomes of microsomal lipids with incorporated PAE (Fig. 6A) at 37°C gave rise to signal B, the intensity of which depends on the concentration of microsomes in the mixture (Fig. 6 B, C). This indicates a transfer of PAE from liposomes to microsomes. Similarly, the addition of small amounts of pure

TABLE II

^{31}P-NMR CHEMICAL SHIFT OF PAE IN DIFFERENT ORGANIC SOLVENTS

The solvents No 5–7 form hydrogen bonds to PAE.

Solvent	Chemical shift (ppm)
1 CH_3COCH_3	31 2
2 C_6H_{12}	31.5
3 CH_3CH	31 4
4 CCl_4	31 6
5. CH_3OH	34.9
6 CH_3COOH	34 8
7 $HCONH_2$	33 9

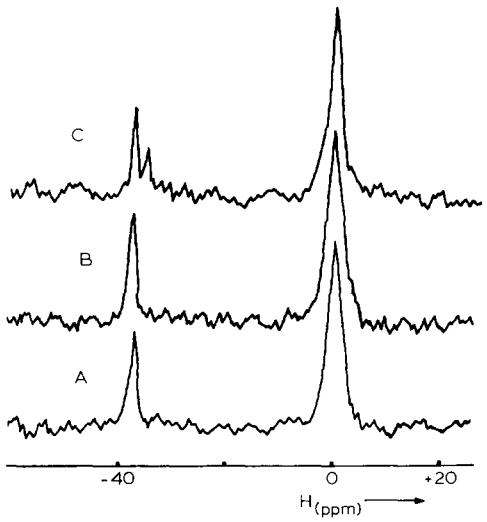

Fig. 8. 81 MHz ^{31}P-NMR spectra of *P*-450 and *P*-450 reductase reconstituted in liposomes of PC (A), of PC/PA = 15 : 0.3 (mol) (B) and of PC/PE/PA = 10 · 5 0 3 (mol) (C) with PAE (protein/PAE = 8 w/w) at 37°C 3000 scans were accumulated for each spectrum

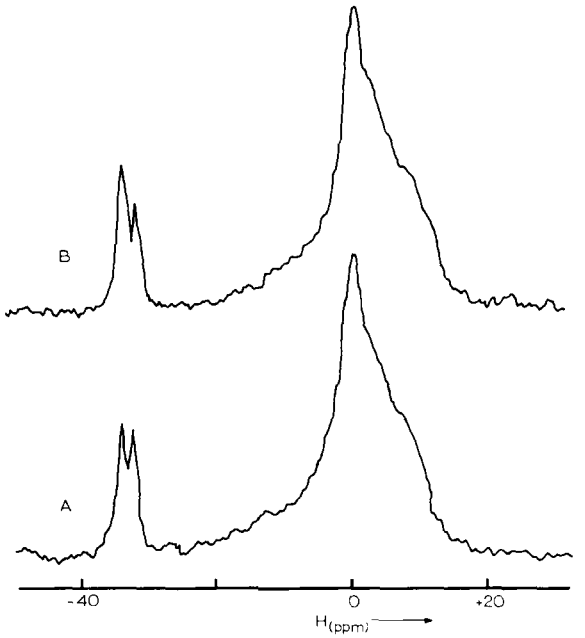

Fig. 9. 81 MHz ^{31}P-NMR spectra of microsomes with PAE (phospholipid/PAE = 28 w/w) at 37°C without (A) and with sodium dithionite (B)

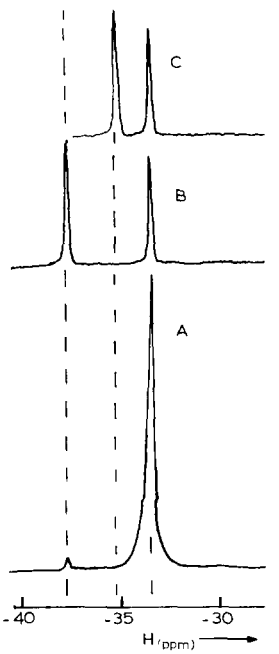

Fig. 10. 81 MHz ^{31}P-NMR spectra of PAE in water at a concentration of 5% (A) and 0.01% (B) by wt. and on addition of hexylamine hydrochloride (C) Spectra (B) and (C) were amplified by a factor of 20 compared to spectrum (A).

liposomes of microsomal lipids (up to 10 wt%) to microsomes with PAE at 37°C increased the intensity of signal B. Liposome concentrations above 10 wt% caused no further increase of the intensity of signal B. In contrast, the addition of pure PC liposomes to microsomes with PAE at 37°C did not influence the intensity of signal B (spectra not shown), thus indicating that a particular phospholipid species of the microsomal lipids (but not PC) is involved in the origin of signal B.

In a further series of experiments the influence of *P*-450 and *P*-450 reductase on signal B in reconstituted systems was investigated. When both proteins were reconstituted in liposomes of microsomal lipids the PAE gave rise to signal A and signal B at 37°C. However, when only *P*-450 (Fig. 7A) or *P*-450 reductase (spectrum not shown) was reconstituted under identical conditions no signal B appeared. Obviously the interaction of the two proteins is essential for the formation of signal B.

The influence of lipids was analyzed by use of reconstituted systems of different phospholipid compositions. Signal B appeared in a reconstituted system with a molar stoichiometry of PC/PE/PA = 10 : 5 : 0.3 whereas no signal B was observed when *P*-450 and *P*-450 reductase were reconstituted in PC liposomes or in PC/PA liposomes (PC/PA = 15 : 0.3, molar ratio) (Fig. 8).

Finally some biologically active substances were examined with respect to the interaction of PAE with microsomes. Benzphetamine as a typical *P*-450 substrate had no effect at concentrations up to 10 mM at 37°C. The addition of Na dithionite caused a decrease of signal B down to 80% (Fig. 9 A, B).

Discussion

The ^{31}P-NMR chemical shift difference of PAE in inert organic solvents such as CCl_4 and in polar solvents such as CH_3OH of about 5 ppm (Table II) is caused by the formation of a hydrogen bond via the phosphoryl group of PAE to corresponding proton donor groups of the polar solvent [21,22]. The extent of the downfield shift of PAE caused by the hydrogen bond depends on its strength [23,24] and is characteristic of each solvent (Table II).

The chemical shift difference between signal A and B (about 2 ppm) indicates two different sites of PAE in microsomes. The shift of signal B on addition of $EuCl_3$ suggests that site B is localized near the preferential binding site of Eu^{3+} ions (pseudo contact shift interaction). It is probable that the presence of PE in the membrane is essential for the formation of signal B. The influence of acidic lipids on the origin of signal B cannot be excluded completely however the small amount of acidic lipids (PA) used in reconstitution experiments (Fig. 8) suggests that this can be only an intermediate influence. Moreover, site B shows saturation characteristics. These facts can be explained by assuming a localization of PAE not far from the PE headgroups. The intensity ratio of signal B to that of the microsomal lipids of about 0.2 at 37°C above the saturation concentration of PAE in site B (Fig. 3) suggests a maximum stoichiometry of about 1 : 1 between PAE and PE in site B. The chemical shift difference between signal A and signal B might be explained by hydrogen bonds of different strengths at both sites. The PAE at site B might be preferentially bound to the PE headgroup (P = O · · · H–N) whereas at site A P = O · · · H–O bonds are preferred. The different strengths of these bonds (the former is weaker than the latter according to Ref. 25) might cause the stronger downward shift of signal A. This assumption is supported by chemical shift measurements of PAE in water. PAE layered on water or dispersed as macromolecular oil drops gives rise to one signal at −33.5 ppm and another one at −37.6 ppm with a far lower intensity (Fig. 10A). A dispersion of very small amounts of PAE makes the two signals comparable in intensity (Fig. 10B). The signal at lower field represents the small amount of hydrogen bonded PAE (P = O · · · H–O) which is identical to signal A whereas the signal at −33.5 ppm is caused by noninteracting PAE. The addition of hexylamine hydrochloride, which possesses the same proton donor group as the PE headgroup, gives rise to an upward shift of the hydrogen bonded PAE to −35.2 ppm (Fig. 10C), at higher hexylamine hydrochloride concentrations the noninteracting PAE is also shifted to −35.2 ppm. This is caused by the formation of P = O · · · H–N bonds with the amine group as proton donor similar to the bonds which give rise to signal B. The preferential interaction of PAE with site B (Fig. 3) or with hexylamine hydrochloride in water might be supported by other factors, such as enthalpy (see below).

A broad phase transition of PE in microsomes in the 15–30°C temperature region has been observed by various methods [5,26]. At the transition to the gel state PAE is squeezed out of the PE-rich regions. Therefore at low temperatures no signal B can be observed in microsomes (Fig. 2A).

Signal A is not influenced by $EuCl_3$ up to concentrations of 30 mM, suggesting that PAE at site A is located far from preferential binding sites of Eu^{3+} ions. It is well established that PAE is incorporated in pure PC multilayers in the interface region of the lipids [8], which enables the PAE molecules to form P = O · · · H–O hydrogen bonds. PAE has an effective molecular cone shape (cross sections of the phosphoryl group larger than that of the alkyl chain) according to Monte Carlo calculations [27]. The hydrophobic interaction of PAE in a bilayer competes with flexoelastic forces caused by the cone shape of the PAE molecules The presence of signal A in the whole temperature range studied suggests that site A is located in lipids such as PC which exhibits no phase transition in this temperature range. The identity of the chemical shift of PAE incorporated in pure PC liposomes with that of signal A proves the validity of this assumption. The lateral diffusion of PAE can be assumed to be faster than that of the lipids, which is supported by Monte Carlo calculations in a model system [28]. This faster diffusion averages the phosphorus chemical shift anisotropies of PAE [29,30] to zero at all temperature studied.

As we did not observe signal B in trypsinized microsomes (Fig. 4) nor in multilayers (Fig. 3) or liposomes of microsomal lipids it must be originate from mutual interactions between proteins, lipids (PE) and PAE. This assumption is supported by the reappearance of signal B on addition of cytochrome *c* to trypsinized microsomes (Fig. 3C). Cytochrome *c* might occupy binding sites of *P*-450 reductase which were split by trypsin, thus functionally reconstituting the reductase *P*-450 interactions with respect to the formation of signal B.

The transfer of PAE to microsomes via PAE containing liposomes (indicated by signal B in Fig. 5 B, C) stressed the role of the proteins and the

higher affinity of site B as compared to site A. The transfer mechanism of PAE between microsomes and liposomes may be explained by fusion of both species, by a fast exchange of mixed micelles of PAE and lipids or by a molecular PAE exchange due to collisions. On the other hand the different actions of liposomes of microsomal lipids and of PC liposomes on microsomes with PAE emphasized not only the importance of the lipids in these interaction, it also excluded that PC is the essential lipid component concerning site B. Therefore the conclusion seems reasonable that PE as second main lipid component of microsomes is essential together with *P*-450 and *P*-450 reductase. Particularly the fact that signal B was observed in a system which contained only small amounts of PA and no PI or PS may exclude that an direct interaction of acidic lipids with PAE contributes to signal B. In general, an interaction of PAE with PS or PI is unlikely due to inability of these lipid headgroups to form strong hydrogen bonds. On the contrary, a preferential PE-PAE interaction in the fluid state is more likely because of the hydrogen bond discussed above and the opposite effective molecular shapes of the two molecules [27,31].

Mutual interactions of proteins, lipid and PAE are explicable by a structural model which is based on the experimentally established existence of clusters of *P*-450 and *P*-450 reductase which are surrounded by a certain lipid. The interaction of this lipid with *P*-450 may influence the functional activity of *P*-450 in the 20–25°C temperature region as reported in Refs. 32–34. In connection with the results reported above and the experimentally established phase transition of PE in this temperature region it seems reasonable to conclude that PE represents this boundary lipid. The dynamical state of such a structure may change with temperature depending on phase transitions of PE and conformational changes of *P*-450. This has been demonstrated recently by the dependence of the lateral diffusion of *P*-450 on the lipid phase state [35].

The isotropic line shape of signal B indicates fast molecular motions of PAE within site B averaging the static interactions to zero. Molecular motions in the region of the NMR timescale are indicated also by the temperature dependent rotational diffusion of *P*-450 as reported in Refs. 2, 3 and 36.

The exchange of PAE between site A and site B is slow ($\tau_{ex} > 1$ ms; τ_{ex}, exchange time estimated from the chemical shift difference of site A and B).

The failure of benzphetamine to influence the intensity of signal B proves that a binding of PAE as a *P*-450 substrate cannot be origin of signal B.

The influence of sodium dithionite on signal B indicates that the state of *P*-450 (reduced or oxidized) is of importance in the PE/PAE interaction at site B.

The same behaviour as reported for PAE was observed for dodecane phosphonic acid diethyl ester in microsomes and liposomes. One can expect that the variety of phosphonic acid esters which were extensively studied in thermotropic systems [8] exhibit a similar behaviour.

We can summarize our results as follows: (1) PAE is incorporated in microsomes at two different sites without influencing the functional activity. (2) By use of PAE as a phosphorus probe molecule a preferential interaction of PE with *P*-450 and *P*-450 reductase was shown which supports the proposal that PE is a boundary lipid of *P*-450 in microsomes.

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