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Interaction of nonionic detergents with phospholipids in hepatic microsomes at subsolubilizing concentrations as studied by ^{31}P-NMR

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The effect of low concentrations of nonionic detergents with different critical micelle concentrations such as Triton X-100, Brij 35 and octylglucoside on rabbit liver microsomes is studied by means of ^{31}P-NMR, ^{1}H-NMR, dynamic light scattering and functional investigations. Hexane phosphonic acid diethyl ester was used as a phosphorus membrane probe molecule to monitor the interaction of detergent molecules with microsomal phospholipids by ^{31}P-NMR. This method is more sensitive than ^{31}P-NMR of phospholipids alone and permitted the estimation of the maximum number of detergent molecules which can be incorporated in microsomes without the formation of mixed micelles outside the membrane. These membrane saturation concentrations were determined to be 0.07 (Brij 35), 0.1 (Triton X-100) and 0.4 (octylglucoside) (molar ratio of detergent/total phospholipids). Above these detergent concentrations, mixed micelles consisting of detergent and membrane constituents are formed, coexisting with the microsomes up to the membrane solubilization concentration. The results indicate a dependence of the membrane saturation concentration on the critical micelle concentration of the detergent and a preferential removal of phosphatidylcholine over phosphatidylethanolamine from the microsomes by all detergents studied.

Introduction

The solubilization of biological membranes and membrane constituents by nonionic detergents is a method widely used for the extraction of proteins and lipids in their native state as well as for reconstitution of proteins in liposomes. The solubilization process on the molecular level at low detergent concentrations is, however, not well understood. Particularly, little reliable information is available about the interaction of detergent molecules with the components of intact biological membranes and about the amount of detergent which can be incorporated into such membranes without influencing their structure and functional activity.

The effect of low detergent concentrations on biological membranes has been studied mainly by means of enzymic techniques [1–6]. Nuclear magnetic resonance has been applied almost exclusively to the investigation of effects of detergents on model membranes [7–11].

^{31}P-NMR is a well-established method for studying membrane structures [12]. However, ^{31}P-NMR of lipds is not sensitive enough to allow a quantitation of the effects of very low detergent concentrations on biological membrnes. This is due to the broad and complex signals exhibited by phospholipids in bilayer arrangement and to the low number of phospholipids interacting with de-

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tergent molecules at the subsolubilization level. Nevertheless, this method has been used recently to follow the solubilization of sarcoplasmic reticulum vesicles [13].

In a previous paper, hexane phosphonic acid diethyl ester (PAE) was established as a phosphorus membrane probe molecule to study protein-lipid interactions in microsomes [14]. The sensitivity of this membrane probe molecule is sufficient to be useful in the detection of detergent-lipid interactions in microsomes by ^{31}P-NMR. The application of this method allowed the estimation of the membrane saturation concentration of the detergents studied in the microsomal membrane. Among the variety of nonionic detergents Brij 35, Triton X-100 and octylglucoside were selected for their wide field of application and the considerable difference in their critical micelle concentrations of more than one order of magnitude. The results obtained by ^{31}P-NMR agree well with dynamic light scattering and ^{1}H-NMR measurements performed on microsomes and mixed micelles. One can expect that the different stages of solubilization of the microsomal membrane upon addition of detergent influence its functional activity. Therefore, the reduction reaction of cytochrome *P*-450 in microsomes was analyzed as a function of the detergent concentration. These investigations prove an unchanged functional behaviour in the membrane saturation concentration range. The onset of solubilization above this concentration as evidenced by NMR and dynamic light scattering causes a decrease of the functional activity of the enzyme system. The initial stage of solubilization of microsomes is preferably related to membrane areas rich in phospholipids such as phosphatidylcholine which are not boundary lipids.

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)/1 mM EDTA (buffer A) to remove pyrophosphate and detached ribosomes. The final pellet was suspended in buffer A which contained 10% deuterium oxide to a final concentration of 22 mg microsomal protein per ml (118 μM cytochrome *P*-450). For ^{1}H-NMR measurements, a $K^2H_2PO_4/K_2{}^2HPO_4$ buffer (pH 7.3) was used instead of buffer A. By determination of the phosphorus content of the microsomes used, a protein/phospholipid ratio of 1.2 (w/w) was obtained. The phospholipid composition was obtained by ^{31}P-NMR according to Ref. 14: 52% phosphatidylcholine, 28% phosphatidylethanolamine, 11% phosphatidylinositol and 9% phosphatidylserine. The protein content, the cytochrome *P*-450 content and the phosphorus content of the microsomes were determined according to Refs. 15, 16 and 17, respectively.

Part of the microsomal suspension in deuterated buffer was treated with Triton X-100 or octylglucoside at different concentrations (incubation time 1 h) and then ultracentrifuged for 1 h at $100\,000 \times g$ at 25°C. The supernatants were studied by dynamic light scattering and after that ^{1}H-NMR measurements were performed on pellets and supernatants. Control measurements with 30 min incubation time yielded identical results.

^{31}P-NMR measurements were performed at 81 MHz under conditions of broad-band inverse gated proton decoupling at 37°C using a Bruker WP-200 spectrometer. 1000 transients were accumulated uisng 90° radiofrequency pulses with a 3 s interpulse time. An external standard (80% H_3PO_4) was used for chemical shift calibration. A 30 Hz line-broadening was applied to the free induction decays. PAE was added to the microsomes at a molar ratio of total microsomal phospholipids: PAE of 6 in all samples studied by ^{31}P-NMR. The detergent was dissolved in buffer A (10 wt% detergent) and added 30 min before measurement under mild stirring. For each detergent concentration a fresh sample of microsomes was used. Control measurements were performed at a lower microsome concentration in the sample (14 mg protein per ml) with identical results concerning the chemical shifts of the two PAE signals.

The PC/PE ratio in the microsomes after detergent treatment was determined using high resolution ^{31}P-NMR. A Bruker AM 250 Spectrometer operating at 101 MHz was used and broad-band inverse gated decoupling, 90% radiofrequency pulses (18 μs) and a 5 s interpulse delay were

applied. 2000 scans were accumulated for each spectrum, giving a reproducibility of the integral intensities of the PC and PE signals as compared to a standard sample of 2%. The PC/PE molar ratio was deduced from this integral intensities. The samples were prepared by incubation of microsomal dispersions in buffer A as described above (22 mg protein per ml) with different detergent concentrations for 1 h. After that, the samples were diluted with buffer A and ultracentrifuged. In order to obtain high-resolved ^{31}P-NMR spectra of the phospholipids, the pellets were completely solublized in buffer A containing 10% (w/w) Triton X-100.

^{1}H-NMR measurements were performed at 200 MHz with a 2.1 s acquisition time at ambient temperature. An internal DSS standard (4,4-dimethyl-4-silapentane sodium sulfonate) was used for chemical shift calibration.

Dynamic light scattering measurements were carried out at 37°C in the homodyne autocorrelation mode as described previously [14]. The samples were cleaned from dust by filtering the suspensions through 400 nm membrane filters (Sartorius, F.R.G.). The microsomal phospholipid concentration in the sample was 1.3 mM. Control measurements as a function of the detergent concentration were performed at a microsomal lipid concentration of 0.7 mM with identical results. For each detergents concentration (incubation time 30 min) a fresh sample was used.

The NADPH-dependent reduction reaction of cytochrome *P*-450 was measured at ambient temperature as reported previously [18]. The cytochrome *P*-450 concentration in the smple was 0.5 μM.

Hepes buffer, Brij 35 and Triton X-100 were obtained from Serva (F.R.G.), octylglucoside was from Calbiochem (U.S.A.), the substances for the deuterated buffer preparation as well as deuterium oxide were purchased from Isocommerz (G.D.R.).

Results and Discussion

The incorporation of PAE into hepatic microsomes at 37°C gives rise to a characteristic ^{31}P-NMR spectrum, consisting of two narrow and symmetrical PAE lines at 37.3 ppm (denoted as signal A) and 35.1 ppm (denoted as signal B) (Fig. 1A). Signal A represents PAE incorporated in membrane areas rich in phosphatidylcholine (PC) and interacting with water molecules via a hydrogen bond. Signal B is caused by PAE hydrogen bonded to the amine group of phosphatidylethanolamine (PE) which interacts as a boundary lipid with cytochrome *P*-450 and *P*-450 reductase [14]. The signal caused by the microsomal phospholipids is almost symmetrical, due mainly to the motional averaging on the NMR timescale by the lateral diffusion of the phospholipids [19].

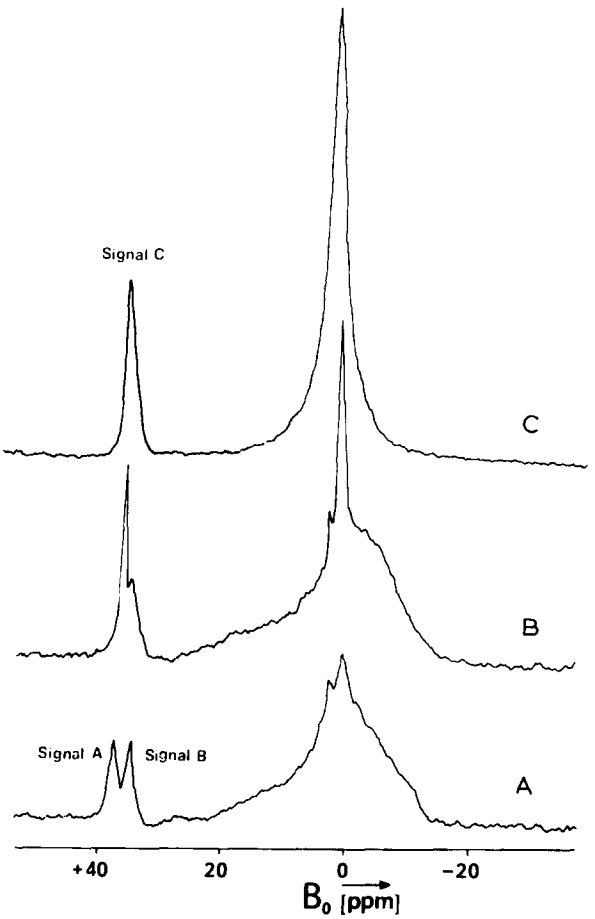

Fig. 1. 81 MHz ^{31}P-NMR spectra of microsomes with PAE at 37°C without detergent (A) and after addition of octylglucoside giving a molar ratio of octylglucoside/total phospholipids = 1.3 (B) and 3 (C).

The addition of Brij 35, Triton X-100 or octylglucoside, at concentrations up to molar ratios of detergent/total phospholipids = 1.1 (Brij 35), 0.6 (Triton X-100) and 1.3 (octylglucoside), to

microsomes with PAE caused a shift of signal A toward 35.1 ppm (Brij 35 and Triton X-100) or 35.5 ppm (octylglucoside) above a definite concentration (Figs. 1B and 2). These chemical shifts were found to be identical to that of PAE solubilized in mixed micelles of the corresponding detergent (which will be denoted as signal C).

Signal B is not shifted upon addition of detergent, as can be observed in the case of octylglucoside (Fig. 1B). In the range octylglucoside/total phospholipids = 1.2–3.0 (mol) there is a coexistence of signal B and signal C and above a molar ratio of 2.2 the intensity of signal B decreases in favour of signal C and ceases to exist at octylglucoside/total phospholipids = 2.8 (mol) (Fig. 1C). This could not be observed for Brij 35 and Triton X-100 because of the near coincidence of the chemical shifts for PAE in Triton X-100 or Brij 35 micelles (signal C) and signal B. The ratio of the integral intensities of the PAE signals and the phospholipid signal remained constant over the whole experiment.

The linear shift of signal A versus the detergent concentration (Fig. 2) can be explained by a fast exchange of PAE between site A, giving signal A in microsomes and mixed micelles formed by detergent, PAE and membrane constituents. Increasing the detergent concentration increases the population of mixed micelles and thus the amount of solubilized PAE molecules. As signal A reaches the position of signal C almost all PAE from site A is solubilized.

As mentioned above, PAE in site A is hydrogen-bonded to water molecules but not to membrane constituents. Therefore it can readily exchange between membrane and mixed micelles. PAE in site B is tightly bound to membrane phospholipids (hydrogen-bonded to PE) and the exchange between site A and site B is slow ($\tau_{ex} > 1$ ms; τ_{ex} is the exchange time estimated from the chemical shift difference in Fig. 1A) [14]. Thus, the contribution of site B to the exchange with mixed micelles can be neglected. This is indicated also by the coexistence of two PAE signals (signal B and C) at higher octylglucoside concentrations (Fig. 1B). Even if this coexistence could not be measured at Brij 35 and Triton X-100 addition because of the almost identical chemical shifts of signals B and C, it seems reasonable to assume that they coexist in a certain concentration region, too.

The ultimate change of signal B in favour of signal C upon addition of octylglucoside is caused by the solubilization of molecules from site B, which gives signal B in mixed micelles.

The narrow signal with a lorentzian lineshape superimposed on the broader microsomal phospholipid signal at −0.5 ppm represents the part of phospholipids solubilized in mixed micelles (Fig. 1B). This signal increases with increasing detergent concentration, indicating that membrane phospholipids are involved in the exchange between membrane and micelles. However, as stated in Ref. 10 for large unilamellar PC vesicles, this exchange is likely to be much slower than that of PAE, at least at low detergent concentrations. Moreover, the sensitivity of the phospholipid signal upon the addition of detergent is less than the chemical shift of signal A in the subsolubilizing detergent concentration range. The first alterations in the phospholipid signal (the occurrence of a sharp isotropic signal superimposed on the broad bilayer signal) were observed at approx. 20% higher detergent concentrations than the beginning of the signal A shift.

According to the three-stage model of solubilization proposed by Lichtenberg et al. [1,4] for model membranes, the following general sequence of events occurs upon the addition of a detergent to a phospholipid bilayer. (1) Incorporation of monomeric detergent molecules into the membrane without any influence on the membrane structure. (2) Upon exceeding a definite detergent concentration in the bilayer called the membrane saturation concentration, the formation of mixed micelles of detergent and membrane constituents occurs. Over a certain concentration range both states (membrane and mixed micelle) coexist. (3) Above some definite detergent concentration all membrane constituents are converted into mixed micelles. This is referred to as membrane solubilization concentration. The latter concentration can be determined readily by turbidity measurements, whereas the membrane saturation concentration is much more difficult to detect [1]. In complex biological systems such as microsomes, which additionally contain a considerable amount of proteins (about 50 wt.% for microsomes), one can

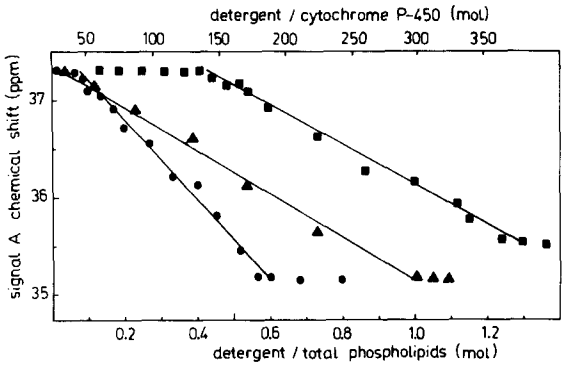

Fig. 2. The ^{31}P-NMR chemical shift of signal A of PAE in microsomes at 37°C versus the detergent concentration for Brij 35 (▲), Triton X-100 (●) and octylglucoside (■).

expect that neither concentration is sharply defined.

Our results can be interpreted in terms of this model. The beginning of the formation of mixed micelles is connected with the onset of a rapid exchange of PAE between membrane and micelles. Thus, the very beginning of the shift of signal A upon addition of detergent represents the membrane saturation concentration of the corresponding detergent in microsomes. From Fig. 2 this saturation concentration can be estimated as a molar ratio of detergent/total phospholipids = 0.07 (Brij 35), 0.1 (Triton X-100) and 0.4 (octylglucoside).

The existence of the membrane saturation concentration in this range is supported by dynamic light scattering and ^{1}H-NMR measurements performed on samples of microsomes treated with Triton X-100 which have been ultracentrifuged to separate micelles and membranes. Two samples with Triton X-100 : total phospholipids = 0.08 (denoted as sample 1) and 0.4 (denoted as sample 2) were ultracentrifuged as described in Materials and Methods. The supernatants were studied by dynamic light scattering, wheras ^{1}H-NMR measurements were performed on pellets and supernatants in order to reveal the composition of the aggregates. In the supernatant of sample 2 mixed micelles with a mean apparent hydrodynamic diameter (d_{app}) of $d_{app} = 18$ nm ($\delta_z = 0.16$) were obtained, whereas no any aggregates of micellar dimensions were detected in the supernatant of sample 1.

By means of ^{1}H-NMR a molar ratio of Triton

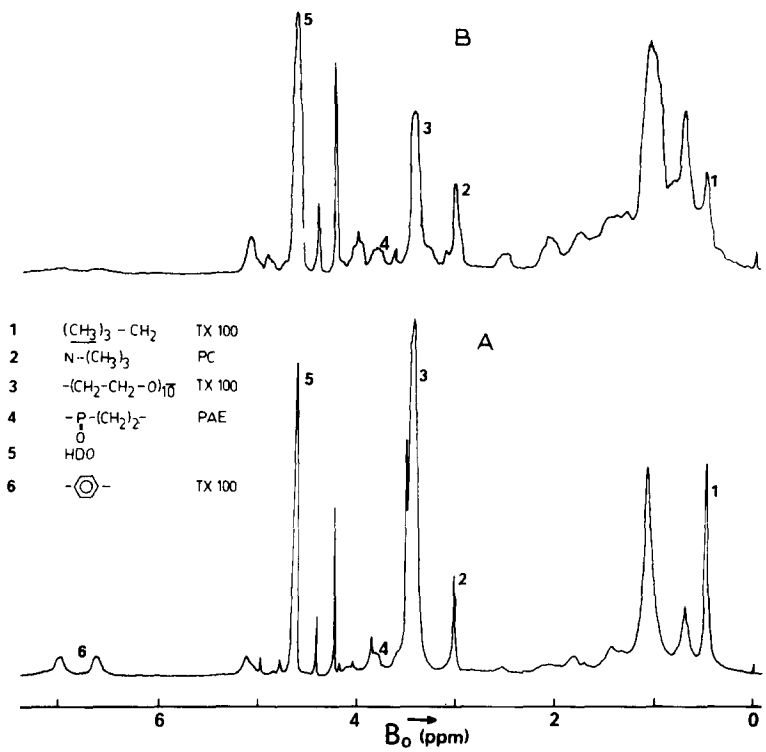

Fig. 3. 200 MHz ^{1}H-NMR spectra of the supernatant (A) and the pellet (B) of microsomes which were treated with PAE and Triton X-100 after ultracentrifugation. Some assignments are given.

X-100/PC/PAE = 3 : 1 : 0.8 was determined in the supernatant of sample 2 using the integral intensities of the signals assigned in Fig. 3A. In the pellet of this sample a ratio of PC/Triton X-100 = 2 (mol) was estimated from the spectrum in Fig. 3B. In contrast, in the supernatant of sample 1 no resonance signals caused by PC or Triton X-100 could be detected, whereas in the pellet a ratio PC/Triton X-100 = 30 (mol) was obtained (spectra not shown). Identical measurements were performed with a sample of octylglucoside/ total phospholipids = 0.35 (mol). Neither mixed micelles nor octylglucoside could be detected in the supernatant by quasi-elastic light scattering and ^{1}H-NMR in this sample.

Further support for the existence of a membrane saturation concentration is provided by dynamic light scattering measurements on microsomes as a function of the detergent concentration Fig. 4 shows d_{app} of microsomes versus the detergent concentration. Above a molar ratio of detergent/total phospholipids of 0.1 (Triton X-100) and 0.4 (octylglucoside), d_{app} decreases linearly with increasing detergent concentration. This is caused by the solubilization of PAE, microsomal lipids and proteins in mixed micelles. The solubilization process increases the polydispersity of the sample (Fig. 4) at intermediate detergent concentrations so that d_{app} becomes irrelevant due to the z-averaging. At high detergent concentrations (molar ratio of detergent/total phospholipids = 2.2 (Triton X-100) and 3.0 (octylglucoside)) a rapid decrease in polydispersity and d_{app} can be observed. This represents the disappearance of the coexistence between microsomes and micelles in favour of the latter. Above this solubilization concentration, all membrane constituents are solubilized in mixed micelles with d_{app} = 20–30 nm. For Brij 35, a solubilization molar ratio of about 3.5 was determined by this method (data not shown). The onset of the linear decrease of d_{app} at low detergent concentrations as mentioned above is in good agreement with the ^{31}P-NMR results concerning the chemical shift of signal A (Fig. 2). Thus one can conclude that this initial solubilization affects mainly PC-enriched membrane areas.

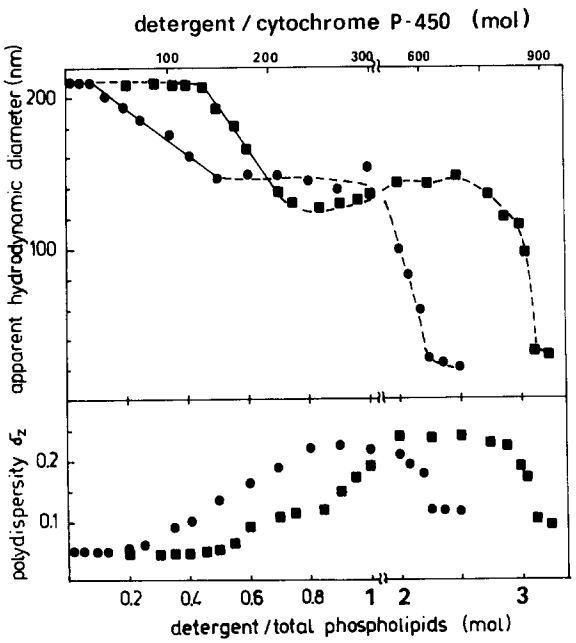

Fig. 4. Apparent mean hydrodynamic diameter and polydispersity parameter of microsomes versus the detergent concentration for Triton X-100 (●) and octylglucoside (■) as measured by dynamic light scattering. The dotted lines indicate concentration regions where d_{app} is considerably shifted to higher values (z-averaging) due to high sample polydispersity.

The octylglucoside concentration at which signal B ceases to exist (octylglucoside/total phospholipds = 2.8 (mol)) in favour of signal C is related to the membrane solubilization concentration of octylglucoside in microsomes. A similar octylglucoside solubilization concentration was measured for PC unilamellar liposomes by Jackson et al. [10]. However, this relation is not sharp. There may be some intact membrane parts present in the solution even if the protein-lipid interaction which accounts for signal B is already damaged. As mentioned above, a somewhat higher solubilization concentration (octylglucoside/total phospholipids = 3.0–3.3 (mol)) was measured by dynamic light scattering.

A preferential removal of PC over PE from the microsomes by the detergents studied is supported by the analysis of the remaining PC/PE ratio of the microsomes after detergent treatment at different concentrations. This has been achieved by performing high-resolution ^{31}P-NMR measurements on microsomal pellets after incubation with detergent. The deduced PC/PE molar ratios as a function of the detergent concentration are shown

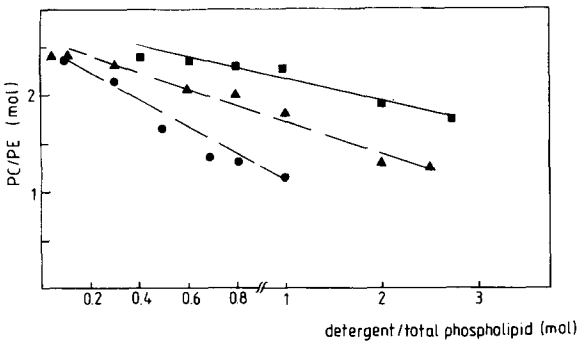

Fig. 5. The remaining PC/PE molar ratios in microsomes after incubation for 1 h with Brij 35 (▲), Triton X-100 (●) and octylglucoside (■) versus the detergent incubation concentration.

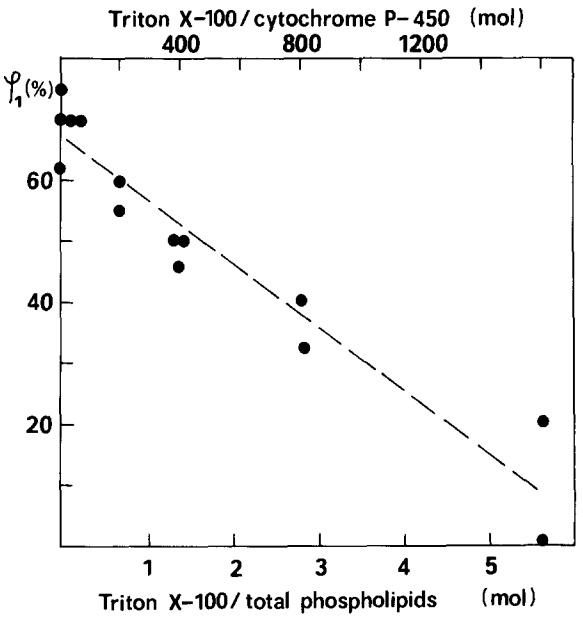

Fig. 6. The amount of the 'cluster' phase reaction (ϕ_1) of cytochrome *P*-450 in microsomes versus tne Triton X-100 concentration.

in Fig. 5. The experiments prove a dilution of PC in the membrane down to about 60% of the initial value (for Triton X-100) over the concentration range studied. The different extent of PC dilution for the three detergents used can be explained by a different partition of the detergent association with microsomal proteins and lipids for each detergent.

In order to study the functional properties which would result from the detergent induced changes as detected by ^{31}P-NMR and light scattering the reduction reaction of cytochrome *P*-450 was analyzed. This reaction represents a distinct tool for the interaction of the main components of the enzyme system cytochrome *P*-450, reductase and lipid [18,20]. A two-exponential treatment of the experimental time function yielded the following characteristics according to the observed solubilization stages (Fig. 6, Table I).

(a) Up to the membrane saturation concentration, the electron transfer properties within the microsomes are unaffected. The phase distribution between 'cluster' and 'random' reduction and the respective rate constants of both processes are nearly unchanged. Thus the functional properties corresponds to the unchanged membrane structure as evidenced by ^{31}P-NMR.

(b) Above the saturation concentration up to the detergent concentration which represents the end of the signal A chemical shift change (Triton X-100/total phosholipids = 0.6), indicating the initial solubilization of PC-enriched membrane areas, a rather small decrease of both kinetic parameters of the 'cluster' phase was observed. Obviously, the solubilization of membrane constituents from the PC-rich membrane areas has no significant effects on the functional behaviour of the enzyme system.

(c) Further increase in the Triton X-100 concentration leads to the disappearance of the rapid reaction phase due to further solubilization of the microsomes, as shown by dynamic light scattering (Fig. 4). This indicates a further perturbation of the protein-lipid interaction which involves pre-

TABLE 1

RATE CONSTANTS, K_1, OF THE 'CLUSTER' PHASE REACTION OF CYTOCHROME *P*-450 IN MICROSOMES VERSUS THE TRITON X-100 CONCENTRATION

Triton X-100/cytochrome *P*-450 (mol)	K_1 (1/s)
30	0.135
190	0.130
380	0.095
760	0.055
1520	0.035
K_1(random)	0.039
K_1(control)	0.134

dominantly lipids with acidic head groups [18] as well as PE [14]. This is consistent with the only minor sensitivity upon the solubilization of PE-enriched areas as reported in (b). The decrease in the signal B intensity in the membrane solubilization concentration range as observed for octylglucoside (Fig. 2) is also consistent with the observed functional characteristics.

In general, one can assume that below the membrane saturation concentration only minor changes occur in the membrane such as fluidization [10], increase of permeability [2] and alterations of some enzymatic activities [3–5].

The considerable differences in the membrane saturation concentrations measured are obviously correlated with the critical micelle concentrations (CMC) of the detergents, which reflects the hydrophobic free energy of transferring a detergent molecule into a micelle (CMC Brij 35 = 0.06 mM [21], Triton X-100 = 0.5 mM [22], octylglucoside = 25 mM [23]). The self association of the corresponding detergent at its CMC results from the entropically unfavourable alkyl chain-water contact. At low detergent concentrations the incorporation of monomeric detergent molecules into the membrane is entropically more favourable and partition between both states (monomeric and membrane bound) is ruled by the hydrophobic free energy of transferring monomeric detergent into the membrne. Additionally, steric interactions between detergent and membrane constituents may contribute to this effect [24]. Monomeric partitioned detergents in the water phase could not be detected by ^{1}H-NMR due to its low concentration.

Above a certain detergent/phospholipid ratio (membrane saturation concentration) the mixed micellar state is the entropically favoured one and become the dominating feature at the membrane solubilization concentration. This is because the interaction free energy of the detergent molecules in micelles reaches a minimum or constant value at some finite aggregation number, whereas in the membrane this energy is only lowered as compared to the monomeric state [25].

But, notwithstanding the correlation with the CMC, the absolute detergent concentration is of minor importance for the membrane state, as already demonstrated in Ref. 10. The measurements with various methods reported in this paper were performed at quite different absolute detergent concentrations but yielded comparable results. In particular, the Triton X-100 concentrations applied in the analysis of the reduction reaction were mainly below its CMC. Moreover, mixed micelles outside the membrane are probably formed at concentrations lower than the corresponding CMC values which were determined in pure binary systems. The molar stoichiometry between detergent and total lipids reflects much better the different solubilization stages.

The slopes in Fig. 2 may be an indication for the solubilization power of the detergent studied. A rapid decrease in the signal A chemical shift with increasing detergent concentration, such as that detected for Triton X-100, indicates an efficient solubilization in accordance with the solubilization concentrations determined by dynamic light scattering and the preference for PC over PE as shown in Fig. 5. This efficiency probably depends on the maximum number of solubilizate molecules (PAE, membrane constituents) which can be incorporated into a micelle of the detergent used. The high solubilization efficiency of Triton X-100 micelles was confirmed very recently in Ref. 26 by fluorescence methods, where a dependence of this efficiency on the micellar shape and the polarity of its interior was claimed.

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