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ACTION OF CHAOTROPIC AGENTS ON DRUG-METABOLIZING ENZYMES IN HEPATIC MICROSOMES

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SUMMARY

The treatment of rat liver microsomes with chaotropic agents such as NaSCN, KI, KNO_3 and urea resulted in a solubilization of a considerable amount of the membrane protein and a decrease in the absorbance of the microsomal suspension. Treatment with NaSCN and KI at 3–4 M concentrations completely abolished microsomal drug oxidation (hydroxylation of aniline and 3,4-benzpyrene); urea and KNO_3 decreased the hydroxylation of aniline and 3,4-benzpyrene by about 40%. Both NADPH– and NADH–cytochrome *c* reductase exhibited a biphasic response: at low concentrations these enzymes were activated by NaSCN and KI, but at higher concentrations an inhibition took place. NADPH cytochrome P-450 reductase was only inhibited by these ions. UDPglucuronyltransferase, catalyzing the conjugation step in drug metabolism, was activated 3–4-fold after treatment of the microsomes with 0.5 M NaSCN or 1.0 M KI; higher concentrations were inhibitory. NaSCN treatment sensitized UDPglucuronyltransferase for trypsin digestion. Weaker chaotropic agents, urea and KNO_3, exhibited lesser effects on drug oxidation and on UDPglucuronyltransferase. However, NADH–cytochrome *c* reductase was particularly activated by these agents. The results obtained support the concept that the oxidizing enzymes are more superficial in the microsomal membrane than is UDPglucuronyltransferase. The location of UDPglucuronyltransferase behind a hydrophobic barrier in the membrane of liver microsomes is supported.

INTRODUCTION

Chaotropic agents such as SCN^-, I^-, NO_3^-, guanidine and urea favour the transfer of apolar components to water by increasing the lipophilicity of water and weakening the hydrophobic bonds of membranes (Hatefi and Hanstein[1]). Chaotropic agents thus provide an effective means for the solubilization of multicomponent enzyme systems as shown in case of mitochondrial enzymes (Davis and Hatefi[2]). Among the forces which contribute to the stability of the microsomal membrane, hydrophobic and electrostatic attractions are most significant (*cf.* Hendler[3]). Chaotropic agents have, however, been used only to a limited extent in the resolution of microsomal enzymes (Spatz and Strittmatter[4]).

Microsomal enzymes carry out drug oxidation, which is an organized multistep process, and subsequent conjugation with glucuronic acid (see Smith and Williams[5]; Conney[6]). The enzymes which catalyze these reactions constitute a significant fraction of the total microsomal protein (Estabrook *et al.*[7]). Cytochrome P-450, the terminal oxidase agent in drug oxidation (Cooper *et al.*[8], Mason *et al.*[9]) is located in the hydrophobic region of the membrane, and it is readily converted to inactive P-420 form by various agents, including neutral salts, urea, and guanidine (Imai and Sato[10]). Microsomal NADPH–cytochrome *c* reductase, when catalyzing the reduction of cytochrome P-450, requires phospholipid for activity (Strobel *et al.*[11]). Microsomes also contain another hemoprotein, cytochrome b_5, which possibly also participates in electron transport reactions in drug oxidation (Hildebrandt and Estabrook[12]). The microsomal NADH–cytochrome *c* reductase activity is coupled as a whole (Siekevitz[13]) or largely (Strittmatter[14]) with the enzymatic system composed of NADH–cytochrome b_5 reductase and cytochrome b_5. Garfinkel[15] has, however, isolated from pig liver microsomes a flavoprotein which exhibits NADH–cytochrome *c* reductase activity but which is different from NADH–cytochrome b_5 reductase and is free from the contamination by cytochrome b_5. UDPglucuronyltransferase, catalyzing the conjugation of the products of the oxidative step, is also tightly bound to the microsomal membrane and its solubilization has proved difficult (Hänninen and Puukka[16], Mowat and Arias[17]).

In order to study the solubilization of the enzymes related to drug metabolism in the microsomal membrane, rat liver microsomes were treated with various chaotropic agents in the present report. The hydroxylation of aniline and 3,4-benzpyrene, the level of NADPH- and NADH–cytochrome *c* reductases, NADPH–cytochrome P-450 reductase and UDPglucuronyltransferase, both from the solubilized and unsolubilized fractions, were measured. The trypsin sensitivity of the UDPglucuronyltransferase was also studied.

MATERIALS AND METHODS

Male Wistar rats (200–250 g) fed *ad libitum* were used. Rats were killed and bled by decapitation. The microsomal fraction was isolated as described (Vainio and Hänninen[18]). The protein concentrations of the microsomal suspensions were determined by the biuret method (Layne[19]).

Liver microsomes (about 20 mg/ml in 0.25 M sucrose) were incubated with different amounts of NaSCN, KI, KNO_3 and urea (all obtained from Merck AG, Darmstadt, Germany) for 15 min at 0 °C in an atmosphere of air. The unsolubilized membrane fraction was recovered by centrifugation at 105000 × *g* for 60 min. In some experiments the pellet was washed by suspending it in 0.25 M sucrose, and centrifuging again at 105000 × *g* for 60 min. The pellet was finally resuspended in ice-cold 0.25 M sucrose. Dialysis of the 105000 × *g* supernatant after NaSCN treatment was carried out for 24 h at 4 °C against 5 l of 25 mM Tris–HCl buffer, pH 7.4. The changes in absorbance of the microsomal suspensions (3 mg/ml) in the presence of various chaotropic agents were measured at 540 nm in a Perkin-Elmer 139 spectrophotometer.

Microsomal NADPH–cytochrome *c* reductase was solubilized by treating the rat liver microsomes (20 mg/ml) with 1% (w/v) deoxycholate (Merck) and sedimenting the unsolubilized fraction at 105000 × *g* for 60 min. Trypsin (Armour Pharm. Comp.,

Eastbourne, Sussex, England) digestion was carried out at 37 °C for 30 min (0.1 mg trypsin/mg of microsomal protein). The digestion was stopped by adding trypsin inhibitor (Worthington Biochemical Corp., New Jersey, U.S.A.) at three times the amount of trypsin.

Cytochrome *c* reductases were assayed by monitoring the NADPH- or NADH-linked cytochrome *c* reduction at 550 nm in a Unicam SP-800 spectrophotometer (Phillips and Langdon[20]) as described earlier (Vainio and Hänninen[18]). Cytochrome P-450 and cytochrome P-420 were measured by the method of Omura and Sato[21]. For this determination, microsomes were diluted with phosphate buffer (final concentration 0.1 M, pH 7.4) to give 1–2 mg of microsomal protein per ml. For the determination of cytochrome b_5, NADH (0.1 mM) was used as a reducing agent.

NADPH–cytochrome P-450 reductase activity was determined by measuring the formation of the cytochrome P-450–CO complex at 450 nm *versus* 475 nm in a Perkin-Elmer 356 two-wavelength double beam spectrophotometer (Gigon *et al.*[22]). The reaction mixture (3 ml) contained about 2 mg per ml of microsomal protein, 100 mM phosphate buffer of pH 7.5 (bubbled with nitrogen before use), 2 mM glucose and 10 I.U. glucose oxidase (from *Aspergillus niger*, Sigma Chemical Co., St. Louis, Missouri, U.S.A.). After achieving anaerobiosis, CO was bubbled through the cuvette for 1 min, and the reaction was initiated by adding 15 nmoles of NADPH (Boehringer).

The microsomal aniline *p*-hydroxylase activity was determined by the method of Imai *et al.*[23]. The hydroxylation of 3,4-benzpyrene (Sigma) was measured as described by Wattenberg *et al.*[24], with slight modification (Gnosspelius *et al.*[25]), in an Aminco-Bowman spectrofluorometer.

The activity of UDPglucuronyltransferase was determined with 0.35 mM *p*-nitrophenol as the substrate and with 4.5 mM UDP-glucuronic acid (ammonium salt, 98%, Sigma) (Hänninen[26]).

RESULTS

The treatment of rat liver microsomes with chaotropic agents released an appreciable amount of microsomal protein into solution. NaSCN and KI at 2 M solubilized about 30–40% of the protein of microsomes (Fig. 1A). Only about 20–25% of the microsomal protein was translocated by urea and KNO_3 at 3 M. When the microsomal fraction was washed with distilled water, 0.25 M sucrose or 0.15 M KCl, 13, 15 and 17%, respectively, of the protein was recovered in the soluble supernatant. The addition of chaotropic agents to a microsomal suspension decreased the absorbance of the microsomal suspension with the release of protein (Fig. 1B). NaSCN and KI were also the strongest agents in this respect.

The treatment of microsomes with NaSCN or KI at about 2 M almost completely eliminated the hydroxylation of aniline and 3,4-benzpyrene. Benzpyrene hydroxylation was somewhat more sensitive than aniline hydroxylation (Figs 2A and 2B). Treatment with 3 M urea or KNO_3 decreased their oxidation by about 40% (Figs 2A and 2B).

When the component reactions of the microsomal mixed-function oxidase were studied, it was found that NaSCN and KI increased 1.5-fold the activity of microsomal NADPH–cytochrome *c* reductase when present in low concentrations (0.1–0.2 M) (Fig. 3A). The activation was, however, followed by an inhibition when

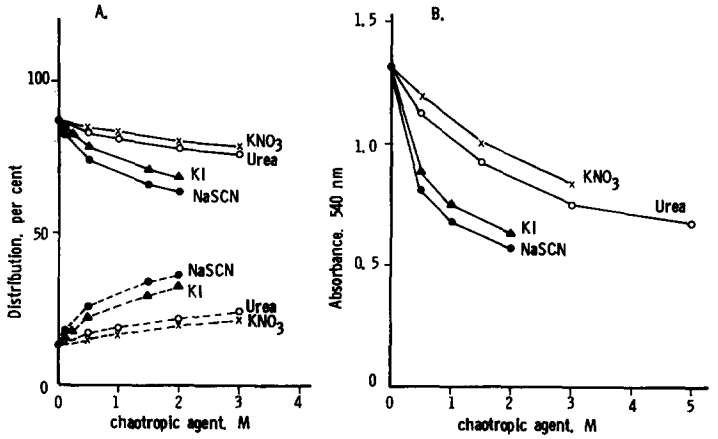

Fig. 1. Release of proteins (A) and changes in the absorbance of the microsomal suspension (B) on treatment of the rat liver microsomes with chaotropic agents. A, The effect of the NaSCN (●—●), KI (▲—▲), KNO_3 (×—×) and urea (○—○) on the distribution of protein between the pellet (———) and supernatant (– – –). B, The effect of various concentrations of NaSCN, KI, KNO_3, and urea on the absorbance of the microsomal suspension at 540 nm (about 3 mg protein/ml).

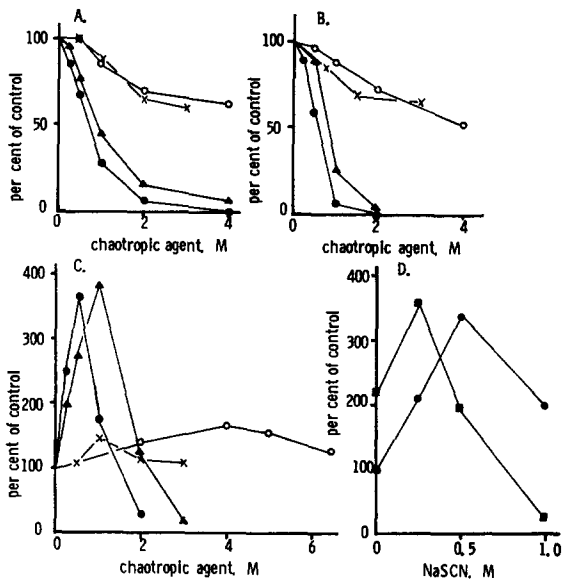

Fig. 2. The effect of chaotropic agent treatment on the activity of aniline hydroxylase (A), 3,4-benzpyrene hydroxylase (B), and UDPglucuronyltransferase (C and D) in the liver microsomes. The points have been calculated as percentages of the activities present in untreated rat liver microsomes (A, 100=0.70 μmoles of *p*-aminophenol formed/min·g of protein; B, 100=0.38 μmoles of 3-hydroxy-3,4-benzpyrene formed/min·g of protein; C, 100=0.32 μmoles *p*-nitrophenol bound/min·g of protein). In D the effect of pretreatment of microsomes with trypsin (■—■) on UDP-glucuronyltransferase activity and the action of NaSCN is illustrated. For symbols see Fig. 1.

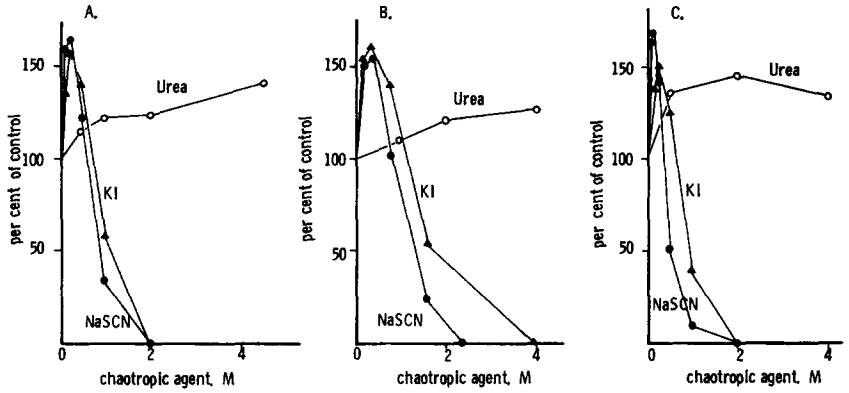

Fig. 3. The effect of various concentrations of chaotropic agents on NADPH$^-$ (A and B) and NADH$^-$ (C) cytochrome *c* reductases. In A and C the activities have been measured from the intact microsomes whereas in B a solubilized NADPH–cytochrome *c* reductase preparation was used. The activities are expressed as percentages of the levels in untreated rat liver microsomes (A and B, 100=126 μmoles cytochrome *c* reduced/min·g of protein; C, 100=1150 μmoles cytochrome *c* reduced/min·g of protein).

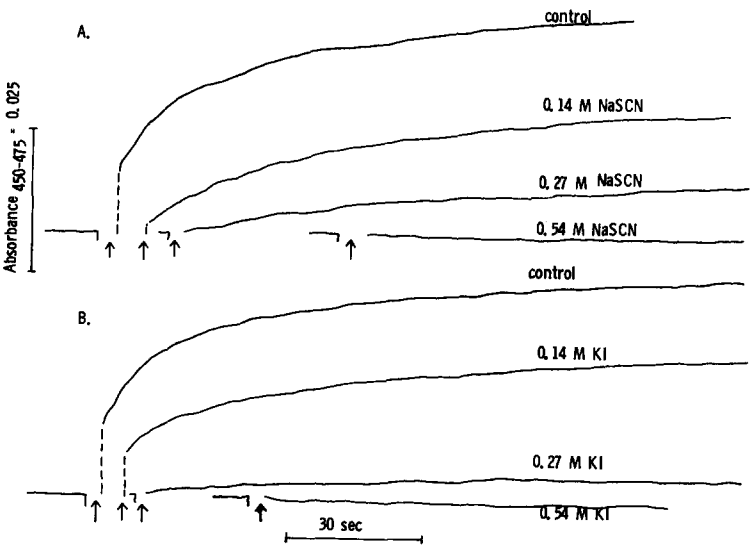

Fig. 4. The effect of various concentrations of NaSCN (A) and KI (B) on the NADPH–cytochrome P-450 reductase. The arrows indicate the addition of NADPH to start the reaction. The amount of microsomal protein added was 5 mg in a final volume of 3 ml.

the concentration was further increased. No NADPH–cytochrome *c* reductase activity could be observed in the presence of 2 M NaSCN or KI (Fig. 3A). A soluble NADPH–cytochrome *c* reductase preparation showed a similar response (Fig. 3B). Inhibition of NADPH–cytochrome P-450 reductase was, however, always observed when NaSCN or KI were added to the incubation medium (Fig. 4). Complete inhibition was found in both cases at about 0.5 M concentration. When urea was added

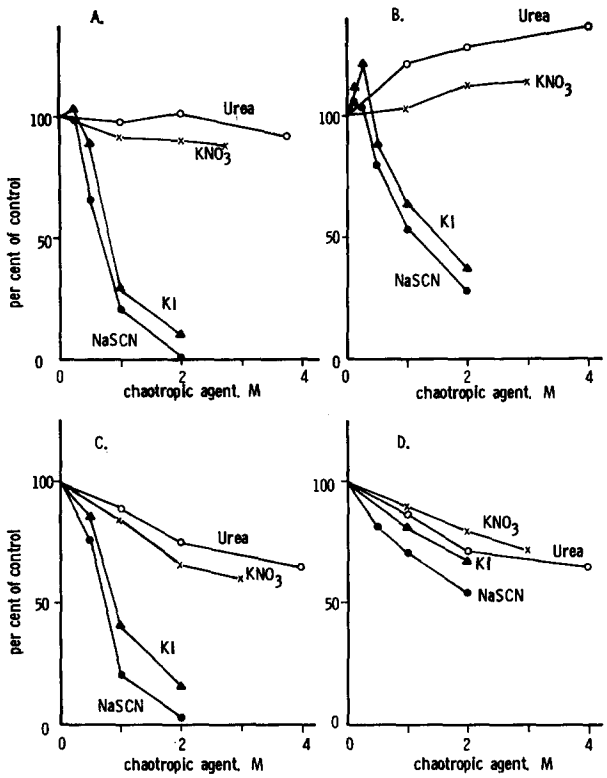

Fig. 5. The effect of the treatment of rat liver microsomes with chaotropic agents on microsomal NADPH-(A) and NADH-(B) cytochrome *c* reductases, cytochrome P-450 (C) and cytochrome b_5 (D). The treated vesicles were pelleted by centrifugation and resuspended in 0.25 M sucrose. The results have been expressed as percentages of the values in the untreated rat liver microsomes (A and B, 100=114 and 1080 μmoles cytochrome *c* reduced/min per g of protein, respectively; C, 100=0.67 μmole/g of protein; D 100=0.45 μmoles/g of protein).

in vitro to the microsomal suspensions, only a slight increase in the microsomal or soluble NADPH–cytochrome *c* reductase could be seen (Fig. 3).

When the microsomes were resedimented after 2 M NaSCN or KI treatment, only minimal NADPH–cytochrome *c* reductase activity could be observed in the pellet and none in the soluble fraction (Fig. 5A). Some NADPH–cytochrome *c* activity (about 10% of the activity in control microsomes) could, however, be recovered from the soluble fraction if it was dialyzed. The treatment of microsomes with urea and KNO_3 had no inhibitory effect on NADPH–cytochrome *c* reductase (Fig. 5A). After the treatment about 15–25% of the NADPH–cytochrome *c* reductase activity present in untreated microsomes was found, however, in the soluble phase. The microsomal NADH–cytochrome *c* reductase was readily solubilized so that about 80% of the activity in untreated microsomes was present in the supernatant after treatment with 3 M KNO_3 (not shown in the figure). The activity in the pellet did not, however, decrease at all, so that the total recovery of NADH–cytochrome *c* reductase was about 190% overall. When 2 M urea was present in the reaction mixture, a 1.5-fold increase in the enzyme activity was observed (Fig. 3C). At low concentrations

(0.1–0.2 M), NaSCN and KI also increased the enzyme activity up to 1.5-fold. High concentrations KI and NaSCN were, however, potent inhibitors of NADH–cytochrome *c* reductase. After treating the microsomes with low KI concentrations some activity was found in the soluble phase, but when the concentration was increased the activity disappeared due to the inhibition of the enzyme.

Chaotropic agents also decreased the content of cytochrome P-450 in rat liver microsomes. It was converted to the P-420 form (Fig. 5C). After treating the microsomes with 2 M NaSCN and KI, almost all of the cytochrome P-450 was in cytochrome P-420 form. The hemoprotein was less sensitive to KNO_3 or to urea treatment (Fig. 5C). Only a minimal amount of the cytochrome was, however, solubilized by the chaotropic agents (5% by 2 M NaSCN).

Cytochrome b_5 was more easily solubilized by the chaotropic agents (Fig. 5D) than was cytochrome P-450. Again, NaSCN was the most active ion, and with 2 M NaSCN about 50% of the cytochrome b_5 was found in the soluble phase. KI also released more cytochrome b_5 from the microsomal membrane than did urea or KNO_3 (Fig. 5D).

Although only an inhibition of drug oxidation was observed by chaotropic agents they proved to be activators of UDPglucuronyltransferase, which catalyzes the conjugation step. The measurable UDPglucuronyltransferase activity increased up to 3–4-fold after 0.5 M NaSCN or 1.0 M KI treatment of microsomes (Fig. 2C). On increasing the NaSCN or KI concentration further, the activity of UDPglucuronyltransferase was progressively inhibited and at 3 M concentration the activity was almost zero. The K_m for the substrate used, *p*-nitrophenol, increased from 1.1 to 2.0 mM, but the K_m for UDPglucuronic acid (1.4 mM) showed no appreciable change after treatment with NaSCN (Fig. 6). The treatment of microsomal membranes with

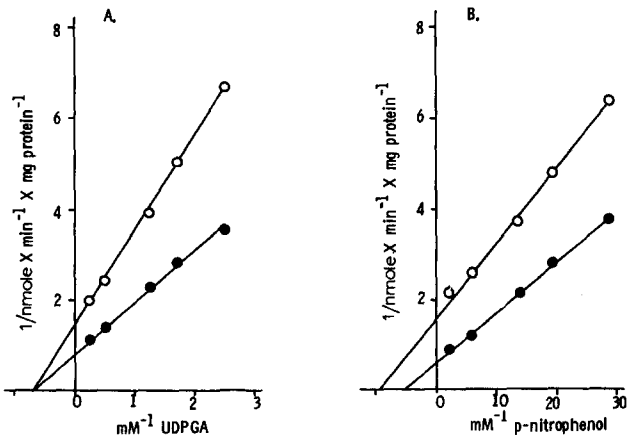

Fig. 6. The effect of NaSCN on the kinetic properties of the microsomal UDPglucuronyltransferase plotted according to Lineweaver and Burk[44]. In A the effect of various UDP glucuronic acid (UDPGA) concentrations on the UDPglucuronyltransferase of the rat liver untreated (○—○) and with 0.5 M NaSCN treated microsomes (●—●) is shown. In B the effect of various *p*-nitrophenol concentrations on the UDPglucuronyltransferase activity has been illustrated, both in untreated microsomes and those treated with 0.5 M NaSCN. The lines have been computed with the least squares method using a PDP-8 computer.

KNO_3 or urea caused also a slight (1.5-fold) activation (Fig. 2C). No inhibition of UDPglucuronyltransferase at the concentrations tested could, however, be observed with KNO_3 or urea. No UDPglucuronyltransferase activity could be observed in the solubilized membrane fraction after treatment with NaSCN or KI either. Pretreatment of microsomes with optimal activating NaSCN concentrations, or higher concentrations sensitized UDPglucuronyltransferase to trypsin digestion. Trypsin digestion however, increased the measurable UDPglucuronyltransferase activity in untreated rat liver microsomes or in those treated suboptimally with NaSCN (Fig. 2D).

DISCUSSION

Drug hydroxylation and subsequent glucuronide conjugation in liver microsomes is catalyzed by a membrane-bound multistep enzyme complex. Chaotropic agents are known to disaggregate the membranes (Hatefi and Hanstein[27]). The polar forces between lipids and proteins are also disrupted when their ionic strengths are increased (Gulik-Krzywicki *et al.*[28]). The destabilization of the membrane structure is followed by a solubilization of some membrane components and an increase in the apparent permeability of the microsomal membrane which decreases the optical density of the microsomal suspension. This explains why chaotropic agents effectively block the microsomal mixed-function oxidase. Furthermore, the chaotropic agents are known to promote the oxidation of membrane lipids (Hatefi and Hanstein[27]), and this oxidation has been shown to interfere with drug oxidation (Orrenius *et al.*[29]).

Of the components of the microsomal mixed-function oxidase, NADPH–cytochrome P-450 reductase was readily inhibited by chaotropic agents. The electron transport from NADPH to cytochrome P-450 thus seems to be highly sensitive to changes in the interaction of these membrane constituents. A compound X, possibly acting as an electron carrier between the flavoprotein and cytochrome, might be responsible for the behaviour observed, since the electron transport to the exogenous acceptor, cytochrome *c*, was even increased by proper concentrations of chaotropic agents. However, the existence of such a compound X in rat liver microsomes is still obscure (see Kimura and Suzuki[30]; Glazer *et al.*[31]; Baron *et al.*[32]). Thus some kind of uncoupling in the microsomal electron transport chain seems to take place between the flavoprotein and cytochrome P-450. NADPH–cytochrome *c* reductase is actually a misnomer, and the enzyme functions in the membrane as NADPH–cytochrome P-450 reductase (Gigon *et al.*[22,33]). Some NADPH–cytochrome *c* reductase was found to be solubilized after treatment with chaotropic agents, probably due to the superficial location of the enzyme in the microsomal vesicle membrane (Ito and Sato[34]; Omura *et al.*[35]; Glazer *et al.*[31]; Masters *et al.*[36]). The antibody studies are in line with the present study in revealing the relatively loose structural association of the flavoprotein and cytochromes in the microsomal membrane (Omura *et al.*[35]; Glazer *et al.*[31]; Masters *et al.*[36]; Baron *et al.*[32]).

Cytochrome P-450 is very sensitive to changes in its environment. The conversion of cytochrome P-450 to cytochrome P-420 results from the disturbance of the hydrophobic environment around the heme (Imai and Sato[10]). Chaotropic agents convert cytochrome P-450 to its inactive P-420 form but do not cause any solubilization. Surfactants, which act primarly on the hydrophobic lipid–protein interactions, are able to solubilize this hemoprotein (Imai and Sato[10]; Lu and Coon[37]; Vainio and

Hänninen[38]). Another microsomal hemoprotein, cytochrome b_5, was translocated into solution to a considerable extent after treatment with chaotropic agents. It has been suggested that cytochrome b_5 is located superficially in the microsomal membrane (Ito and Sato[34]; Omura *et al.*[35]). Contradictory results have been published on the membrane location of NADH–cytochrome *c* reductase (Ito and Sato[34]; Omura *et al.*[35]; Archakov *et al.*[39]), perhaps due to the different reduction mechanisms (see Siekevitz[13]; Strittmatter[14]; Garfinkel[15]; Schulze *et al.*[40]). In the present study, a significant part of the activity was, however, found in the supernatant, which suggests that the enzyme complex might have a superficial location in the membrane.

Although the oxidation step in drug metabolism by liver microsomes was impaired after treatment with chaotropic agents, the conjugation step was enhanced. *In vivo* the two steps take place coupled to each other and conjugated metabolites are mainly excreted (Smith and Williams[5]). Thus, chaotropic agents also uncouple the connection between the oxidation and conjugation steps. The K_m of UDPglucuronyltransferase for the aglycone, *p*-nitrophenol, increased, which is perhaps a reflection of a change in membrane conformation.

The permeability of the vesicle membrane was probably increased, since the chaotropic agent treatment sensitized microsomal UDPglucuronyltransferase to trypsin digestion and caused a solubilization of some (surface) proteins. A treatment of microsomal membranes with a surfactant, digitonin, which is known to increase the UDPglucuronyltransferase activity about 10-fold, has also been shown to sensitize UDPglucuronyltransferase to trypsin digestion (Hänninen and Puukka[41]). These findings are in line with the view that UDPglucuronyltransferase is covered by other membrane components (Hänninen and Alanen[42]; Hänninen and Puukka[16]).

For a multitude of reactions the potency of order of various chaotropic agents is essentially the same, *i.e.*, they follow the order known as Hofmeister's lyotropic series of ions (SCN^-, I^-, NO_3^-, *etc.*, Hofmeister[43]). SCN^- is the most denaturating, depolymerizing and solubilizing anion (Hatefi and Hanstein[1]). In the present study it was found that the more potent a compound is as a chaotropic agent, the more it solubilizes microsomal proteins, the more active it is as an inhibitor of microsomal drug oxidation and the more it activates UDPglucuronyltransferase. Both the first and the second phase in microsomal drug metabolism were found to be sensitive for the action of chaotropic agent, the conjugation step being more resistant and even activated under certain concentrations.

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