EFFECTS OF NEUTRAL SALTS ON HEPATIC MICROSOMAL DRUG-METABOLIZING ENZYME SYSTEM IN RATS

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Abstract—Effects of neutral salts on the drug-metabolizing enzyme system were measured in hepatic microsomes of rats *in vitro*. Aminopyrine N-demethylation was markedly enhanced by Li_2SO_4 , Na_2SO_4 , and K_2SO_4 . Salts such as LiCl , NaCl , and KCl caused an enhancement of the demethylation following by an inhibition at high concentrations. KBr , KI , and KSCN always inhibited the demethylation. Aniline hydroxylation, on the contrary, was not stimulated by the sulfates, and all other salts inhibited the hydroxylation with increasing concentration. The effectiveness of the neutral salts on changing aminopyrine or aniline oxidation activity followed Hofmeister's lyotropic series of ions: $\text{SCN}^- > \text{I}^- > \text{Br}^- > \text{Cl}^- > \text{SO}_4^{2-}$ as anions and $\text{Li}^+ > \text{Na}^+$, K^+ as cations. KSCN , KI and KBr caused both the conversion of cytochrome P-450 to cytochrome P-420 and the inhibition of NADPH cytochrome *c* reductase activity; however, all other salts used in these experiments showed no change of those components. Enhancement of aminopyrine N-demethylation by the sulfates was reversible. It was concluded that cytochrome P-450 associated with aminopyrine N-demethylation is different from that of aniline hydroxylation in the hydrophobic environment of microsomes, and sulfate or chloride causes an enhancement of only cytochrome P-450 activity associated with the demethylation.

Recently, the isolation of the drug-metabolizing enzyme inhibitor from Habu snake venom has been studied in our laboratory (1). During these studies, the eluent obtained from ion exchange column chromatography was used for evaluating the inhibitory action of drug-metabolizing enzymes of rat hepatic microsomes *in vitro*. We found that NaCl , which was added to the elution buffer of the chromatography in order to increase ionic strength, tended to stimulate aminopyrine N-demethylation, but tended to inhibit aniline hydroxylation. This finding seems to conflict with the view that neutral salts inactivate microsomal cytochrome P-450 by the conversion to cytochrome P-420 as reported by Imai and Sato (2). Since the existence of multiple forms of

cytochrome P-450 has been demonstrated in hepatic microsomes (3-5), it was suggested that neutral salts cause a different action on each cytochrome P-450.

We conducted these studies to clarify the effects of neutral salts on the hepatic microsomal drug-metabolizing enzyme system using various salts *in vitro*.

Materials and Methods

Chemicals: Nicotinamide adenine dinucleotide phosphate and glucose-6-phosphate were purchased from the Sigma Chemical Co., St. Louis MO; aminopyrine from Daiichi-Seiyaku Ltd., Tokyo; glucose-6-phosphate dehydrogenase and reduced nicotinamide adenine dinucleotide phosphate from Oriental Yeast Ltd., Tokyo. Acetylacetone and aniline

were purified by distillation, and p-aminophenol was purified by sublimation. All other chemicals were of analytical reagent grade.

Preparation of microsomes: Male Wistar rats weighing 150 to 300 g (from Nihon rat Ltd., Saitama) were used. Animals were fed with a commercial diet (Oriental Yeast Ltd.) and water ad lib. Hepatic microsomes were prepared as follows: The animal was decapitated, and the liver was removed after perfusion with 1.15% KCl solution in situ. The liver was homogenized in a teflon-glass homogenizer with 3 vol. of ice-cold 1.15% KCl solution. The homogenate was centrifuged at 9,000 g for 30 min, and the supernatant fraction was further centrifuged at 105,000 g for 60 min. The resulting microsomal pellet was then resuspended in 1.15% KCl solution and used as the microsome preparation. The microsomes were either used immediately or stored at -20°C for two or three days. No effect of storage on the results was observed.

Analytical methods and assay of microsomal components: The reaction mixture for the assay of aminopyrine N-demethylation and aniline hydroxylation contained microsomes (0.5 to 0.7 mg protein), 80 mM Tris-HCl buffer (pH 7.4), NADPH-generating system (0.33 mM NADP, 8 mM glucose-6-phosphate, 6 mM MgCl_2 , and 0.2 units of glucose 6-phosphate dehydrogenase), and 1 mM substrate in a final volume of 1.0 ml. When necessary, the salts were added to the mixture. The reaction mixture was incubated in a water bath shaker at 37°C under air. The activity of the demethylation of aminopyrine and the hydroxylation of aniline were determined by measuring the formation of formaldehyde and p-aminophenol using the Nash method (6) and the method of Brodie and Axelrod (7) as modified by Imai et al. (8), respectively. Cytochrome P-450 and NADPH cytochrome c reductase were measured by the method of Omura and Sato

(9), and the method of Mazel (10), respectively. The difference spectra were determined using a Shimadzu (MPS-50L) spectrophotometer. Microsomal protein was determined by the method of Lowry et al. (11), using bovine serum albumin as the reference protein. All assays and experiments were done at least in duplicate.

Results

Effects of salts on the activity of aminopyrine N-demethylation and aniline hydroxylation: The treatment of microsomes with neutral salts caused an enhancement or inhibition of aminopyrine N-demethylation as shown in Fig. 1. The salts such as Na_2SO_4

Fig. 1. Effects of salts on aminopyrine N-demethylation. Microsomal aminopyrine N-demethylation activity was measured as described in Materials and Methods using 1 mM aminopyrine and 0.7 mg microsomal protein at 37°C for 20 min. Control activity (no salt) was 46.91 ± 6.80 nmoles formaldehyde/mg protein/20 min. Na_2SO_4 : ●, K_2SO_4 : ⊙, Li_2SO_4 : ⊖, LiCl : ⊗, KCl : △, NaCl : ▲, KBr : ⊙, KSCN : ⊕.

and K_2SO_4 markedly enhanced the aminopyrine N-demethylation with increasing concentrations, and the demethylation activity reached 335% of the control by Na_2SO_4 and 250% by K_2SO_4 at 0.4 M. In the case of Li_2SO_4 , there was marked enhancement of the demethylation, but little increment at concentrations above 0.2 M. A biphasic effect on aminopyrine N-demethylation was observed when LiCl, NaCl, or KCl was added. These salts enhanced the demethylation at low concentrations, but inhibited it at high concentrations above 0.4 M. KBr decreased the demethylation activity dose-dependently. KI and KSCN markedly inhibited the demethylation activity, which was completely lost above 0.2 M of the salts. Some salts at higher concentrations disturbed the color development of formaldehyde, but these effects were all corrected for. The time course of aminopyrine N-demethylation in the presence or absence of salts is presented in Fig. 2. The slope of the curve for aminopyrine

Fig. 2. Time course of aminopyrine N-demethylation. Conditions were the same as described in Fig. 1. All salts were added to the incubation mixture at 0.2M final concentration, except for KSCN at 0.05M. Na_2SO_4 : ●, Li_2SO_4 : ○, LiCl: ⊗, NaCl: ▲, KCl: △, Control: ○, KBr: ●, KSCN: ⊗.

N-demethylation was larger in the case of sulfate or chloride and was smaller in the case of bromide or thiocyanate than that of the control. Figure 3 shows the effects of salts on aniline hydroxylation. Contrary to the marked enhancement of aminopyrine N-demethylation by the addition of sulfate, little increase of aniline hydroxylation was observed by treatment with sulfate, and the hydroxylation activity was about 112% of the control even at 0.6 M Li_2SO_4 . All other salts dose-dependently inhibited the hydroxylation and the order of the inhibition was $KSCN > KI > KBr > NaCl > KCl > LiCl$. This order followed the classical Hofmeister series of ions: $SCN^- > I^- > Br^- > Cl^- > SO_4^{--}$ as anions (12). This effect of salts on aniline hydroxylation was clarified by observing the time course of aniline hydroxylation.

Fig. 3. Effects of salts on aniline hydroxylation. Experimental conditions were the same as described in Fig. 1, except aniline (1 mM) was used as substrate. Control activity (no salt) was 11.01 ± 0.55 nmoles p-aminophenol/mg protein/20 min. Na_2SO_4 : ●, K_2SO_4 : ○, Li_2SO_4 : ○, LiCl: ⊗, NaCl: ▲, KCl: △, KBr: ●, KI: ○, KSCN: ⊗.

Fig. 4. Effects of salts on cytochrome P-450. Aliquots of 4.8 ml microsomes (1–2 mg/1 ml of 0.1 M phosphate buffer, pH 7.4) were incubated with 3.2 ml of various salts at 37°C for 20 min, aerobically. The 8 ml reaction mixture was divided into two cuvettes, and then the CO-difference spectra of dithionite-treated microsomes were determined by the method of Omura and Sato (9). The control value (no salt) was 0.616 ± 0.07 nmoles cytochrome P-450/mg protein. Na₂SO₄: ●, K₂SO₄: ⊙, Li₂SO₄: ⊖, LiCl: ⊗, KCl: Δ, NaCl: ▲, KBr: ●, KI: ⊕, KSCN: ⊗.

Effects of salts on cytochrome P-450: Cytochrome P-450 was determined after incubation of microsomes with salts at 37°C for 20 min, and the results are shown in Fig. 4. The microsomal cytochrome P-450 content was not significantly changed by incubation with sulfate and chloride. Only KBr slightly converted cytochrome P-450 to cytochrome P-420 at 0.8 M, but almost all the cytochrome P-450 was converted by the same concentration of KI and KSCN.

Effects of salts on NADPH cytochrome *c* reductase: Figure 5 shows NADPH cytochrome *c* reductase activity after incubation with salts at 37°C for 20 min. KI and KSCN decreased the reductase activity to 67% and to 5% of the control, respectively,

Fig. 5. Effects of salts on NADPH cytochrome *c* reductase. Aliquots of 1.6 ml microsomes (0.1–0.2 mg/1 ml 0.1 M phosphate buffer, pH 7.4) were incubated with 0.4 ml of various salts at 37°C for 20 min, aerobically. After incubation, 0.5 ml of the microsomes was used for the determination of NADPH cytochrome *c* reductase according to the method of Mazel (10). Control activity (no salt) was 309.23 ± 92.13 nmoles cytochrome *c* reduced/mg protein/min. Na₂SO₄: ●, K₂SO₄: ⊙, Li₂SO₄: ⊖, LiCl: ⊗, KCl: Δ, NaCl: ▲, KBr: ●, KI: ⊕, KSCN: ⊗.

at a concentration of 0.4 M. All other salts had no effect on the reductase activity at the same concentration as KI and KSCN.

Reversible effects of sulfate on the enhancement of aminopyrine N-demethylation: Microsomes were washed by centrifugation after preincubation with 0.4 M Li₂SO₄ or Na₂SO₄ at 37°C for 30 min. Table 1 shows the activity of aminopyrine N-demethylation of washed microsomes in the presence or absence of 0.4 M Li₂SO₄. The activity of aminopyrine N-demethylation was increased to 201% by readdition of 0.4 M Li₂SO₄ in control microsomes. In preincubated microsomes with Li₂SO₄ and Na₂SO₄, the aminopyrine N-demethylation was enhanced to 190% and to 235% by the readdition of

Table 1. Reversible effect of Li_2SO_4 and Na_2SO_4 on aminopyrine N-demethylation enhancement

Preincubation	Formaldehyde (nmoles/mg/30 min)	
	Control	Readdition of Li_2SO_4
Control	57.92±2.19 (100±3.7)	116.89±0.95 (201.8±1.6)
0.4M Li_2SO_4	62.80±0 (100±0)	122.36±6.44 (194.8±10.25)
0.4M Na_2SO_4	62.86±1.35 (100±2.1)	148.33±2.38* (235.9±3.8)

Preincubation was carried out at 37°C for 30 min in a mixture of the following composition: microsomes (4.2 mg/ml, 1.15% KCl), 0.6 ml; 0.2M Tris-HCl buffer (pH 7.4), 0.9 ml; and H_2O or Na_2SO_4 , Li_2SO_4 (0.4M final concentration), 1.5 ml. After preincubation, the microsomes were washed by centrifugation at 105,000 g for 60 min, and resultant pellets were resuspended with the same volume of initial mixture. Aminopyrine N-demethylation activity of washed microsomes was measured as described in Fig. 1 in the presence or absence of 0.4M Li_2SO_4 . Values presented are the average±standard error of triplicate incubations. * $P<0.001$ as compared to the control.

Li_2SO_4 . In the later case, the enhancement of the demethylation was significant. Thus the action of sulfate on enhancing aminopyrine N-demethylation was reversible.

Discussion

In studying the effects of neutral salts on the conversion of cytochrome P-450, Imai and Sato (2) showed that the efficiency of various salts in causing conversion of cytochrome P-450 to cytochrome P-420 followed Hofmeister's lyotropic series of ions (12): $\text{SCN}^- > \text{I}^- > \text{Br}^- > \text{Cl}^- > \text{SO}_4^{--}$ as anions and $\text{Li}^+ > \text{Na}^+$, K^+ as cations. They explained that the conversion of cytochrome P-450, which was caused by various reagents including neutral salts, resulted from the disturbance of the hydrophobic environment around the heme of the cytochrome P-450. Since cytochrome P-420 is incapable of hydroxylating a substrate (13), it is expected that neutral salts decrease drug-metabolizing enzyme activity in the microsomes. As presented in Figs. 1 and 3, however, neutral salts showed both stimulatory and inhibitory effects on drug-metabolizing enzymes. Aminopyrine N-demethylation was markedly enhanced by sulfate and moderately by

chloride. Aniline hydroxylation, on the other hand, was not increased by sulfate; and only inhibition of the hydroxylation was observed by chloride, bromide, iodide, and thiocyanate. The effectiveness of the various ions on altering the activities of aminopyrine N-demethylation and aniline hydroxylation also followed the Hofmeister series of ions. We measured whether the conversion of cytochrome P-450 was caused under the same conditions as for the determination for aminopyrine and aniline oxidation activities. At the maximum concentration of 0.8 M of the salts in our experiments, cytochrome P-450 was almost completely converted to cytochrome P-420 by KI and KSCN and slightly converted by KBr, but no conversion of cytochrome P-450 was observed by sulfate and chloride. Concerning the content of cytochrome P-450 and drug-metabolizing enzyme activity, it was demonstrated that aminopyrine N-demethylation and aniline hydroxylation were decreased to 55% and to 42% of the control by 0.8 M of LiCl, respectively, in spite of no conversion of the cytochrome P-450. Thus aminopyrine N-demethylation and aniline hydroxylation activities were not parallel to the content of

cytochrome P-450. These results, therefore, show that the oxidative activity of cytochrome P-450 can be changed by neutral salts prior to the conversion of the cytochrome P-450 to cytochrome P-420. The change of oxidative activity in microsomes may possibly come from the alteration of NADPH cytochrome c reductase because the reductase is thought to be the rate-limiting step in the drug-metabolizing enzyme system (14). Our results, however, showed no change of NADPH cytochrome c reductase activity after treatment of microsomes with sulfate and chloride. Hence, it is likely that the enhancement or inhibition of drug-metabolizing enzyme activity by those salts may be due to the effect on cytochrome P-450 itself, but not due to the changing NADPH cytochrome c reductase activity.

There are a number of evidences for existence of multiple forms of cytochrome P-450 which have an overlapping activity for various substrates (15, 16). This broad substrate-specificity of cytochrome P-450 is considered to be due to an oily, hydrophobic pocket of cytochrome P-450 which provides a remarkable non-specific substrate binding site (17). On the other hand, aminopyrine and aniline are classified as type I and type II substrates based on the substrate-induced difference spectrum of cytochrome P-450 (18). It is well established that a type I substrate causes spectral change by binding to the hydrophobic region of the cytochrome P-450, and a type II substrate-induced spectrum is due to the interaction of the substrate with the heme iron of the cytochrome (19). Thus the hydrophobic environment of cytochrome P-450 in microsomal membranes is important for aminopyrine N-demethylation. The fact that sulfate or chloride causes a different effect on aminopyrine N-demethylation and aniline hydroxylation suggests that cytochrome P-450 associated with aminopyrine N-demethylation

differs from that of aniline hydroxylation in the hydrophobic environment around its active site, and it suggests that these salts alter such a hydrophobic environment resulting in easy entrance of aminopyrine to the active site. These effects by sulfate or chloride may be the stabilization of cytochrome P-450 associated with aminopyrine N-demethylation as presented by von Hippel and Wong who explained that sulfate and phosphate anions can stabilize ribonuclease due to fit and potentiate the iceberg structure which develops around nonpolar residues of the protein in its unfolded form (20). Further study of the enhancement of aminopyrine N-demethylation by sulfate is in progress.

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