way or to effects of morphine at other sites within the CNS is unclear. It is possible that the two effects of morphine, enhanced dopamine metabolism and impulse flow, reflect activation of opiate receptors at two unrelated sites, and the amount of dopamine released will reflect the sum of these two phenomena. To separate these sites of action will be difficult because of the multiplicity of opiate agonist effects in the extrapyramidal system [13, 15, 16, 18].

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REFERENCES

1. K. Kuschinsky, *Arzneimittal-Forsch.* **26**, 563 (1976).
2. B. Celsen and K. Kuschinsky, *Naunyn-Schmeideberg's Archs Pharmac.* **284**, 159 (1974).
3. N-E. Andén and M. Grabowska-Andén, *J. Pharm. Pharmac.* **30**, 732 (1978).
4. M. C. Nowycky, J. R. Walters and R. H. Roth, *J. Neural Transm.* **42**, 99 (1978).
5. E. Costa, D. L. Cheney, C. C. Mao and F. Moroni, *Fedn Proc.* **37**, 2408 (1978).
6. K. Kuschinsky and O. Hornykiewicz, *Eur. J. Pharmac.* **26**, 41 (1974).
7. P. A. Shore, B. A. McMillen, H. H. Miller, M. K. Sanghera, R. S. Kiser and D. C. German, in *Catecholamines: Basic and Clinical Frontiers* (Eds. E. Usdin, I. J. Kopin and J. Barchas), p. 722. Pergamon Press, New York (1979).
8. P. A. Shore, *J. Pharm. Pharmac.* **28**, 855 (1976).
9. J. R. Walters and R. H. Roth, *Naunyn-Schmeideberg's Archs Pharmac.* **296**, 5 (1976).
10. G. F. Murphy, D. Robinson and D. F. Sharman, *Br. J. Pharmac.* **36**, 107 (1969).
11. N. H. Neff and E. Costa, *Life Sci.* **5**, 951 (1966).
12. J. S. Hong, H-Y. T. Yang and E. Costa, *Neuropharmacology* **16**, 451 (1977).
13. H. Pollard, C. Llorens-Cortes and J. C. Schwartz, *Nature, Lond.* **268**, 745 (1977).
14. G. Calderini, A. Consolazione, S. Garattini and S. Algeri, *Brain Res.* **146**, 392 (1978).
15. F. Moroni, E. Peralta, D. L. Cheny and E. Costa, *J. Pharmac. exp. Ther.* **208**, 190 (1979).
16. H. Simantov, M. J. Kuhar, G. W. Pasternak and S. H. Snyder, *Brain Res.* **106**, 189 (1976).
17. A. C. Cuello and G. Paxinos, *Nature, Lond.* **271**, 178 (1978).
18. K. Gale, F. Moroni, K. Kumakura and A. Guidotti, *Neuropharmacology* **18**, 427 (1979).
19. D. C. German, H. Harden, M. K. Sanghera, D. Mann, R. S. Kiser, H. H. Miller and P. A. Shore, *J. Neural Transm.* **44**, 39 (1979).
20. J. C. Schwartz, H. Pollard, C. Llorens, B. Malfroy, C. Gros, Ph. Pradelles and F. Dray, in *The Endorphins* (Eds. E. Costa and M. Trabucchi), p. 245. Raven Press, New York (1978).

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Effect of ethanol administration on free proline and glutamate in the intact rat liver

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The concentration of free proline is considered to be significant in the formation of collagen [1, 2]. Earlier we observed that the synthesis of proline was increased during the incubation of liver homogenates obtained from ethanol-treated rats or of liver slices when ethanol was added *in vitro* [3]. The conclusions on the role of glutamate as a precursor of free proline in liver are variable [1, 3, 4].

In humans, Kershenobich *et al.* [5] demonstrated a correlation between the concentrations of free proline and collagen in liver. Siegel *et al.* [6] studied alcoholics and did not find any change from normal in serum proline but, following ethanol load, an elevation of proline was found. Similarly, Shaw and Lieber [7] observed in baboons that after a long-term administration of ethanol, proline was depressed in post-prandial serum. Mezey *et al.* [8] observed in rat liver an increase of free proline after ethanol feeding. In our own study [9] we also found that a long-term administration of ethanol to the rats increased the concentration of free proline in liver, although by 10–12 per cent only, on average. Mørland *et al.* [10] did not observe any change in hepatic free proline in rats after long-term ethanol feeding, while Forsander (personal communication) found a decrease in liver proline after a 2-month treatment with ethanol, although there was an increase in liver glutamate.

The purpose of this work was to find out whether a single-dose administration of ethanol will cause an increase of free proline in liver *in vivo*, and to discover what are the simultaneous changes in liver glutamate. There is a shift in the redox balance towards the reducing side during the catabolism of ethanol in liver [11]. This change favours the synthesis of proline. Therefore, we wanted to see whether by diminishing this change in the redox balance caused by the oxidation of ethanol we could prevent the increase in the amount of proline. For that purpose the rats were treated with 4-methylpyrazole, a potent inhibitor of alcohol dehydrogenase [12]. Attempts to influence the redox balance were made also by giving methylene blue to the rats.

Experimental

Treatment of animals. Male Sprague–Dawley rats, aged 2–4 months, and fasted for about 40 hr, were given ethanol by stomach tube, usually 2 g per kg body wt as a 25% (v/v) aqueous solution. In the preliminary experiments, ethanol doses of 4 and 8 g per kg were also used. The control rats received corresponding volumes of water.

4-Methylpyrazole was injected i.p. 10 min before the administration of ethanol. On the basis of the preliminary experiments with doses from 10 μmoles to 2.44 mmoles per kg body wt, the dose adopted for use was 0.2 mmoles or 16.4 mg per kg.

Methylene blue was given 5 or 10 mg per kg as an i.p.

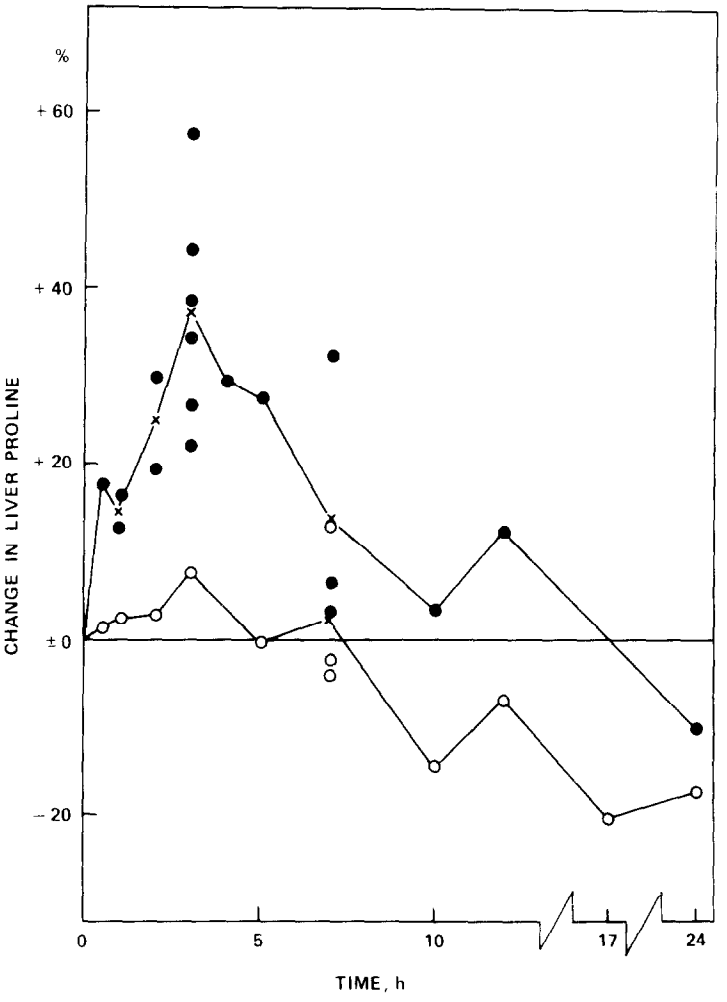

Fig. 1. Change in the concentration of liver proline as a function of time between the administration of ethanol and the killing of rats. The number of experiments at each point of time varies from 1 to 6, and every circle represents the average value from 2 rats. For the details see the Experimental section. The change is calculated as per cent of the value of the corresponding control rats without ethanol. -●-●-●- Rats that received ethanol, 2 g per kg body wt.; -○-○-○- rats that received 4-methylpyrazole, 16.4 mg, and ethanol, 2 g per kg body wt.

injection of an aqueous solution 10 min before the administration of ethanol.

The animals were killed by dislocation of the cervical vertebrae at various time intervals from 0.5 to 24 hr. The livers were immediately dissected and dropped into liquid nitrogen. The time required for the killing of rats and removal of livers was 30 sec to 1 min. It was noted that up to 5 min the speed of this performance had no effect on the level of proline and only slightly affected the level of glutamate. The frozen livers were chopped to smaller pieces and divided into portions so that each sample contained material from every lobe. The samples were kept frozen until used for analyses.

Determination of proline. Frozen liver samples were homogenized in cold Ringer–bicarbonate buffer. Liver homogenate was deproteinized with 75% (v/v) ethanol and centrifuged. The ethanol was evaporated from the supernatant, and proline in the residue was determined according to the photometric method to Troll and Lindsley [13] using a ninhydrin reagent. For the removal of the interfering amino acids, Amberlite IRC-50 resin was used instead of Permutit.

Determination of glutamate. Frozen liver samples were homogenized in cold 75% (v/v) ethanol, centrifuged, and ethanol was evaporated from the supernatant. The residue was dissolved in water, and glutamate was determined according to the method of Borys *et al.* [14], based on the procedure of Graham and Aprison [15]. Glutamate was oxidized to α-ketoglutarate by glutamic dehydrogenase and the fluorescence of NADH formed was measured with a Zeiss spectrophotometer PMQII, excitation 365 nm, emission 470 nm.

Results

Concentrations of proline and glutamate, and the effect of fasting of the rats. The concentration of liver proline in fasted control rats (N = 15) was 0.53 ± 0.016 (S.E.M.) μmoles per g wet wt. The concentration of liver glutamate was 3.42 ± 0.32 μmoles per g wet wt.

The fasted animals were employed to ensure the optimal absorption of ethanol and the homogeneity of their nutritional state. Although fasting of rats for about 40 hr caused an increase in liver proline, the increase was the same in

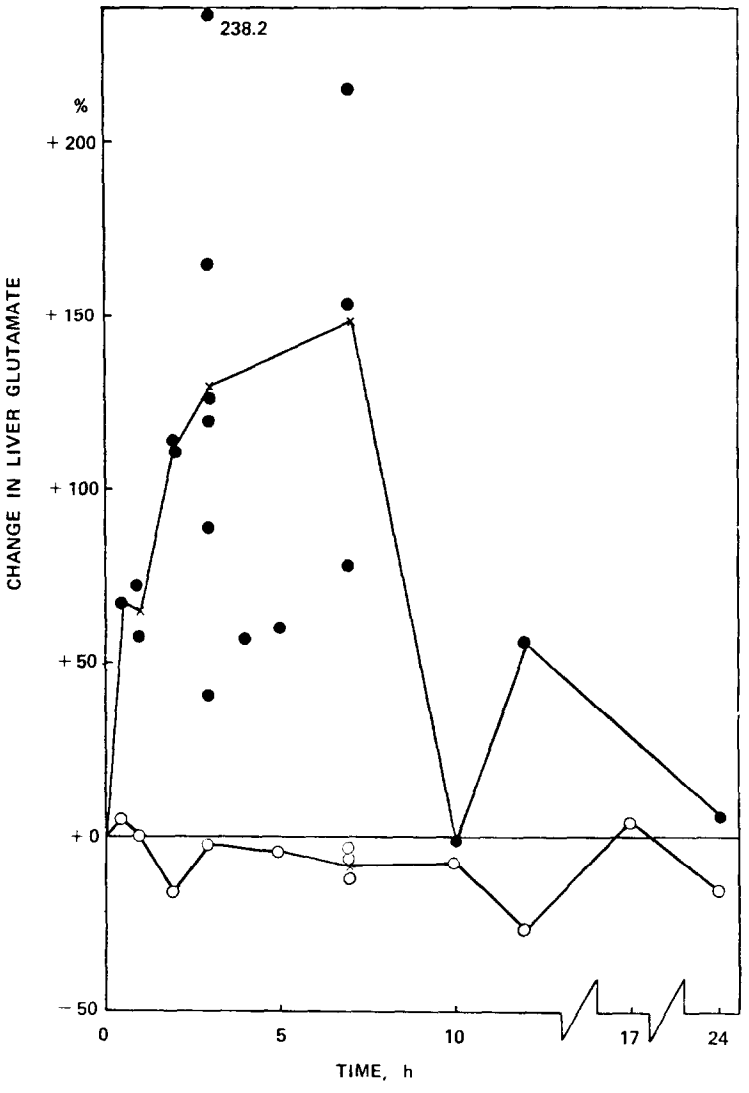

Fig. 2. Change in the concentration of liver glutamate as a function of time between the administration of ethanol and the killing of rats. For the explanations see the legend of Fig. 1.

both control (+ 21.8 per cent) and ethanol-treated rats (+ 21.3 per cent), and thus did not influence the change caused by the administration of ethanol.

Effect of various doses of ethanol. The increase in the concentration of liver proline, measured 2 hr after the administration of ethanol, was almost the same whether the rats had received ethanol 2 g (+ 20 per cent) or 4 g per kg body wt (+ 23 per cent) The corresponding changes in the concentration of liver glutamate were + 85 and + 83 per cent. The dose 8 g ethanol per kg body wt caused a higher rise (+ 113 per cent) in the concentration of proline. However, since the dose 8 g per kg is close to lethal, and there was no difference in the effects of the two lower doses, only 2 g ethanol per kg was used in all following experiments.

Time dependency of the effect of ethanol. The increase in the concentration of proline was at its highest, + 37.3 per cent, 3 hr after the administration of ethanol (Fig. 1). The level of proline returned to almost normal in 10 hr.

The concentration of glutamate increased even more than that of proline, and the maximum level, + 149 per cent, was reached 7 hr after the administration of ethanol (Fig. 2).

When all the points in the period 0.5–7 hr are included, the increase caused by ethanol is significant at the level $P < 0.001$ in both proline and glutamate concentration.

Influence of 4-methylpyrazole on the effect of ethanol. 4-Methylpyrazole, injected 10 min before the administration of ethanol, prevented the increase of both proline (Fig. 1) and glutamate (Fig. 2). At the time points of maximum ethanol-induced increase, at 3 hr for proline and at 7 hr for glutamate, the changes in the concentrations of these metabolites in 4-methylpyrazole-treated rats were + 7.8 and −8.1 per cent, respectively. The effect of 4-methylpyrazole in the ethanol-treated rats was significant at the level $P < 0.001$, whether the values in the period 0.5–7 hr were calculated as independent or non-independent samples. During the latter part of the experimental period the concentration of proline decreased below the control level. The injection of 4-methylpyrazole alone did not change appreciably the level of proline (± 0.0 per cent) and glutamate (−1.8 per cent) in the livers of control rats.

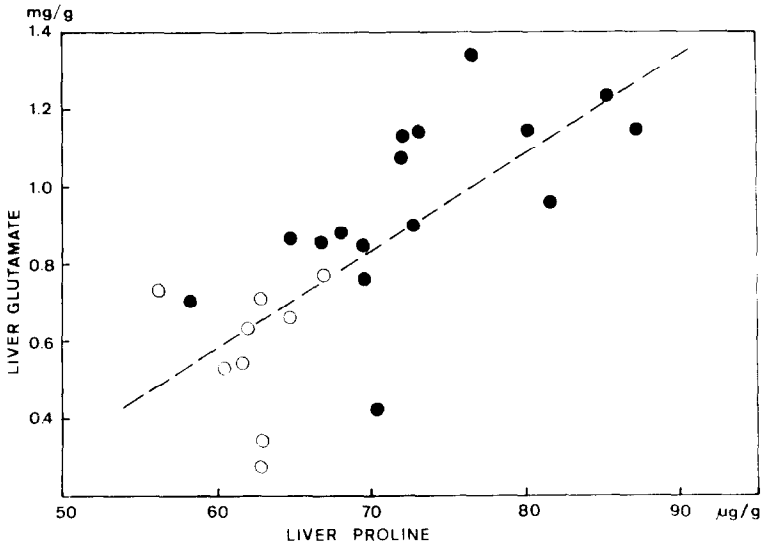

Fig. 3. Ratio of the concentration of liver glutamate to the concentration of liver proline. The concentrations are expressed in mg and in μg per g liver wt, respectively. The calculated regression line (please see the text) is presented. ○ Control rats; ● ethanol-treated rats.

Ratio of glutamate to proline. When the concentrations of glutamate are plotted against the concentrations of proline from the same liver samples (Fig. 3), there is a moderately high linear correlation: $y = 0.025\,x + 0.917$, $r = 0.731$, $P < 0.001$. For ethanol-treated rats separately the corresponding parameters are $y = 0.019\,x + 0.426$, $r = 0.628$, $P < 0.01$, but in the group of control rats no correlation is present ($r = -0.0046$).

Influence of methylene blue on the effect of ethanol. Methylene blue had no effect on liver glutamate of control rats, but increased the concentration of liver proline (+ 34 per cent). In ethanol-treated rats the injection of methylene blue caused a 25 per cent additional increase in liver proline and a 23 per cent additional increase in liver glutamate. The combined effect of ethanol and methylene blue was a 74 per cent increase in liver proline and a 94 per cent increase in glutamate.

Discussion

The increase in the concentration of free proline in liver found after the administration of ethanol was in accordance with our expectations. The concentration of glutamate increased even more, but that does not necessarily indicate a precursor–product relationship. In an earlier work [3] we have found that the conversion of [^{14}C] glutamic acid to proline in liver slices was increased by 39–46 per cent when ethanol (20–50 mM) was added to the medium. Rojkind and Diaz de León [1] have observed only slight conversion of glutamic acid to proline (0.03 μmole/g at a 3 hr incubation) by liver slices from normal rats, but an 8-fold amount (0.24 μmole/g) by slices from rats made cirrhotic by the administration of CCl_4.

The prevailing condition of liver plays a role also in the incorporation of proline into collagen. For example, ethanol *in vitro* caused a significant increase in the incorporation of proline in the biopsy specimens of alcoholic hepatitis or active cirrhosis, but had no effect on the collagen synthesis by normal or fatty liver [16]. In the rats on the high-fat, low-protein diet, the effect of ethanol on the collagen synthesis was never more marked than in the rats on the normal diet [9].

The levels of liver proline and glutamate depend on the time point after the administration of ethanol (Figs. 1–2) [6]. Therefore, one wonders whether the liver samples studied by Kershenobich *et al.* [5] were taken under the catabolism of ethanol. Furthermore, during the first weeks of long-term administration of ethanol to the rats, liver proline reaches a maximum and then levels off (unpublished results).

Ethanol has also been reported [17] to lower the amount of labelled proline incorporated into collagen. There was, nevertheless, an increase in the amount of collagen, which was attributed to the inhibition of collagen breakdown by ethanol.

In a study of collagen biosynthesis in the chick embryo [4], glutamate was found not to be a major precursor of free proline in the leg minces; arginine and ornithine, instead, were active precursors of both free proline and free glutamate. This conclusion was confirmed in this laboratory by means of rat liver preparations (Forsander, Lukkari and Mikkonen, personal communication).

The metabolism of liver proline is influenced also by the simultaneous urea synthesis. The urea synthesis utilizes the amino groups liberated by glutamate dehydrogenase as well as the carbon skeleton of glutamate for the formation of ornithine. This catabolic outlet of glutamate through the ornithine cycle is reported to be suppressed by ethanol [18]. This explains, in part, the accumulation of glutamate and proline in liver during the ethanol catabolism.

When the effects of the oxidation of ethanol on the redox balance are prevented by a simultaneous administration of 4-methylpyrazole, only slight changes are observed in the concentrations of proline and glutamate in liver. This indicates that the metabolism of these amino acids is not affected by ethanol as such but through its catabolism.

It is postulated that the reduced form of methylene blue predominates in the tissues *in vivo* [19]. This form would support the reducing reactions. An accentuation of the effects of ethanol on liver proline and glutamate is indeed observed after the treatment of rats with methylene blue.

In addition to the stimulation of the biosynthesis of proline, the mode of action of ethanol on the proline metabolism could be the inhibition of the oxidation of proline in liver to Δ-pyrroline-5-carboxylate [20], the inhibition of gluconeogenesis and urea synthesis from proline [21, 22], or changes in the intracellular proline concentration through the effect of ethanol on the cell membranes.

Many redox steps are involved in the metabolism of proline. The oxidation of proline is NAD-dependent but, in the reduction of glutamate to proline, NADP-dependent dehydrogenases are involved. Since the NAD-dependent reactions are more affected by ethanol than the NADP-dependent reactions [23], the oxidation of proline is thus in all probability inhibited more than its formation is promoted [24].

In summary, a single dose of ethanol caused a significant but temporary increase in the concentration of both proline and glutamate in the rat liver, maximally + 37 and + 149 per cent, respectively. The concentrations of glutamate and proline had a linear correlation. When 4-methylpyrazole was given simultaneously with ethanol there were no changes in the concentrations of proline and glutamate. The administration of methylene blue corroborated the effects of ethanol.

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REFERENCES

1. M. Rojkind and L. Diaz de León, *Biochim. biophys. Acta* **217**, 512 (1970).
2. G. A. M. Finerman, S. Downing and L. E. Rosenberg, *Biochim. biophys. Acta* **135**, 1008 (1967).
3. H.-M. Häkkinen and E. Kulonen, *Biochem. Pharmac.* **24**, 199, (1975).
4. S. Zinker and M. Rojkind, *Conn. Tissue Res.* **1**, 275 (1972).
5. D. Kershenobich, F. J. Fierro and M. Rojkind, *J. clin. Invest.* **49**, 2246 (1970).
6. F. L. Siegel, M. K. Roach and L. R. Pomeroy, *Proc. natn. Acad. Sci. U.S.A.* **51**, 605 (1964).
7. S. Shaw and C. S. Lieber, *Am. J. clin. Nutr.* **28**, 423 (1975).
8. E. Mezey, J. J. Potter, R. J. Slusser and W. Abdi, *Lab. Invest.* **36/2**, 206 (1977).
9. H.-M. Häkkinen, K. Franssila and E. Kulonen, *Scand. J. clin. Lab. Invest.* **35**, 753 (1975).
10. J. Mørland, R. Flengsrud, H. Prydz and L. Svendsen, *Biochem. Pharmac.* **28**, 423 (1979).
11. O. Forsander, *Biochem. J.* **105**, 93 (1967).
12. H. Theorell, T. Yonetani and B. Sjöberg, *Acta chem. scand.* **23**, 255 (1969).
13. W. Troll and J. Lindsley, *J. biol. Chem.* **215**, 655 (1955).
14. H. K. Borys, D. Weinreich and R. E. McCaman, *J. Neurochem.* **21**, 1349 (1973).
15. L. T. Graham, Jr. and M. H. Aprison, *Analyt. Biochem.* **15**, 487 (1966).
16. T. S. N. Chen and C. M. Leevy, *J. Lab. clin. Med.* **85**, 103 (1975).
17. K. S. Henley, E. G. Laughrey, H. D. Appelman and K. Flecker, *Gastroenterology* **72**, 502 (1977).
18. J. B. Field, H. E. Williams and G. E. Mortimore, *J. clin. Invest.* **42**, 497 (1963).
19. N. Blass and D. Fung, *Anesthesiology* **45**, 458 (1976).
20. M. Rojkind and D. Kershenobich, in *Progress in Liver Diseases*, Vol. 5 (Eds. H. Popper and F. Schaffner), p. 294. Grune & Stratton, New York (1976).
21. E. Fellenius, H. Carlgren, and K.-H. Kiessling, *Life Sci.* **13**, 595 (1973).
22. H. E. S. J. Hensgens, A. J. Meijer, J. R. Williamson, J. A. Gimpel and J. M. Tager, *Biochem. J.* **170**, 699 (1978).
23. R. L. Veech, R. Guyn and D. Veloso, *Biochem. J.* **127**, 387 (1972).
24. O. A. Forsander, *Proc. Int. Med. Symp. on Alcohol and Drug Dependence, ICAA Symposium in Japan*, p.182. Tokyo-Kyoto (1977).

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Inhibition of hepatic metabolism of azathioprine by furosemide in human liver *in vitro*

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The immunosuppressive drug azathioprine undergoes thiolysis to 6-mercaptopurine, a biotransformation important for the ultimate action of the drug [1, 2]. This conversion is catalyzed by glutathione (GSH) S-transferase [3] predominantly in the liver [4]. *In vivo* studies in rats have shown inhibition of hepatic metabolism of azathioprine by endogenous and exogenous glutathione S-transferase inhibitors, bilirubin and probenecid, respectively [5]. After kidney transplantation, azathioprine is often used together with the diuretic drug furosemide, which can inhibit GSH S-transferase activity in the kidney [6]. Since the liver seems to play the dominant role in the glutathione mediated metabolism of azathioprine [4], it was of interest to study whether furosemide could also inhibit the formation of 6-mercaptopurine in this tissue. Such an interaction could possibly result in a diminished immunosuppressive effect of azathioprine. Therefore we have studied furosemide inhibition of the formation of 6-mercaptopurine in the liver *in vitro*. We used human liver from our human liver bank [7] since marked species differences in the metabolism of xenobiotics exist.

Eight specimens of human adult liver were obtained within 20 min after stopping artificial respiration and life supporting treatment of patients without cerebral activity who were selected as kidney donors. Homogenization and subcellular fractionation was initiated shortly thereafter, as described elsewhere [7]. The 100,000 *g* supernatant fraction was stored at −80° until used. Relevant patient data are depicted in Table 1. Some donors received drugs the last few days before death. However, usually they received only single (e.g. during anesthesia) or a few doses, and we believe that this pre-mortem treatment did not markedly influence the capacity of the liver to metabolize drugs.

The GSH-S-transferase activity with azathioprine in the 100,000 *g* liver 'cytosol' fractions was determined according to methods previously described [3]. The final reaction mixture (3.0 ml) consisted of azathioprine (0.1 or 0.2 mM) dissolved in 0.1 M sodium phosphate buffer, pH 6.5, 0.17 mM reduced glutathione and 200 μl of liver cytosol. The reaction was measured by the production of the 317 nm product (6-mercaptopurine) in a Beckman ACTA MVI spectrophotometer at 37° with the reference containing