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Effect of repeated oral administration of isosorbide dinitrate on hepatic glutathione S-transferase activity in the rat

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Glyceryl trinitrate, isosorbide dinitrate and other alkyl nitrates are denitrated by glutathione-dependent soluble liver enzymes in the rat [1, 2] and other species [3, 4]. The enzyme(s) concerned was known as nitrate ester reductase (EC 1.8.6.1), but this name should be abandoned as a result of the elegant work of Jakoby *et al.* [5], who purified the glutathione S-transferases (EC 2.5.1.18) to homogeneity and also showed that these enzymes were involved in the denitration of alkyl nitrates, such as glyceryl trinitrate and isosorbide dinitrate [6, 7].

The induction of microsomal drug-metabolising enzymes by certain compounds and the subsequent clinical implications have been extensively studied [8–10] but there is little known regarding the inducibility of the GSH S-transferases by their own substrates.

The influence of repeated oral administration of isosorbide dinitrate on the activity of microsomal and soluble liver enzymes is reported in this paper.

MATERIALS AND METHODS

Isosorbide dinitrate (ISDN), 1,4:3,6-dianhydro-D-glucitol dinitrate, was kindly provided by Sanol-Arzneimittel Dr. Schwarz GmbH, Monheim, Germany. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase and nicotinamide adenine dinucleotide phosphate ($NADP^+$) were obtained from the Boehringer Corporation, London, U.K. 1,2-Dichloro-4-nitrobenzene and *trans*-benzylideneacetone were supplied by Koch–Light Laboratories Ltd., Colnbrook, U.K. Ethylmorphine hydrochloride B.P.C. was obtained from MacFarlan Smith Ltd., Greenford, U.K. Glyceryl trinitrate was available as a 1% (v/v) ethanolic solution. Glutathione (GSH) was obtained from Schwarz–Mann, Orangeburg, NY, U.S.A.

Animal experiments. Twenty-four male CFY rats (a strain of Sprague–Dawley origin) of *ca.* 150 g body wt, were obtained from Anglia Laboratory Animals, Huntingdon, U.K. The animals received food (Standard Laboratory Diet No. 1, Spratts, Barking, U.K.) and tap water *ad libitum*. After an acclimatisation period of 4 days, the animals were allocated randomly into one control and two test groups, each group consisting of eight animals. The test rats received isosorbide dinitrate (10 mg/kg/day or 100 mg/kg/day), dissolved in corn oil (0.5 ml) daily by oral intubation during 28 days and control rats received corn oil (0.5 ml/rat) daily during the same period. Doses were administered during the mid-afternoon. The chosen doses were suitable multiples (about 5 × and 50 ×) of a likely daily dose of the drug in humans.

At 20 hr after the final daily dose, the rats were weighed, and sacrificed by cervical dislocation. The livers were removed immediately, weighed, and placed in ice-cold 0.1 M—K-orthophosphate buffer, pH 7.4, containing KCl (1.15%, w/v). All further operations were carried out at 4°C. The livers were homogenised separately in orthophosphate-buffered isotonic KCl solution (4 vols), pH 7.4, in an electrically-driven glass Potter–Elvehjem-type homogeniser with a Teflon pestle. The homogenates were centrifuged at 10,000 *g* for 20 min at 4° and the supernatant decanted. Microsomal fractions were prepared by centrifugation of the supernatant at 105,000 *g* for 1 hr at 4° (MSE Superspeed 65 Ultracentrifuge). The supernatant (post—105,000 *g* supernatant) was decanted, dialysed [11] and stored at −20° until used for the assay of soluble (cytosol) enzymes. The microsomal pellet was suspended in orthophosphate-buffered KCl solution, pH 7.4, using a hand-operated glass Potter–Elvehjem-type homogeniser, so that 1 ml of the suspension was approximately equivalent to 300 mg liver (wet wt). The microsomal suspension was kept at 4° for use in enzyme assays and cytochrome P450 estimations, which were performed within 2 hr.

Drug-metabolising enzyme activity. Cytochrome P450 concentrations [12], aniline hydroxylase [13] and ethylmorphine *N*-demethylase [14] were measured as indicators of microsomal enzyme activity. As an indicator of soluble enzyme activity, GSH S-transferases were measured using 1,2-dichloro-4-nitrobenzene [15], benzylideneacetone [16] and glyceryl trinitrate as substrates. Activity towards the alkyl nitrate was measured by incubating GSH (24 mM), glyceryl trinitrate (40 mM) and dialysed rat liver supernatant in 0.2 M-orthophosphate buffer, pH 6.5, at 25° for 5 min. The reaction was terminated by the addition of acetic anhydride (0.4 ml) followed by 1% (w/v) sulphanilamide in 20% (v/v) HCl (2 ml). After mixing, aqueous 0.05% (w/v) naphthylethylenediamine hydrochloride (2 ml) was added and the absorbance of the resulting solution measured at 540 nm after 15 min. Enzyme activity was calculated by reference to a standard curve constructed from sodium nitrite taken through the above procedure [17]. Protein concentrations were measured by the method of Lowry *et al.* [18] using bovine serum albumin as a standard.

RESULTS AND DISCUSSION

Body and organ weights. Repeated oral administration of isosorbide dinitrate did not significantly affect the body or organ wt of rats compared to controls. During 28 days, body wt increased by a mean of about 148%. At sacrifice the mean wt of the liver, kidneys, and lungs of rats accounted for about 4, 0.8, and 0.5 per cent respectively of their body wt.

Protein concentrations. Repeated oral administration of isosorbide dinitrate did not significantly affect hepatic microsomal protein concentrations in rats compared to controls (Table 1). However, hepatic soluble protein concentrations in rats dosed at 10 mg/kg/day were significantly lower ($P < 0.05$) than controls (Table 2).

Hepatic enzyme activity. Repeated oral administration of isosorbide dinitrate did not significantly affect the activity of the microsomal enzymes measured (Table 1), except that concentrations of cytochrome P450 were significantly lower ($P < 0.01$) in rats dosed at 100 mg/kg/day. The mean activity of ethylmorphine *N*-demethylase in the microsomes from rats dosed at this level was lower, but

Table 1. Effect of repeated oral administration of isosorbide dinitrate on hepatic microsomal drug-metabolising enzyme activity

Parameter*	Controls	Treated 10 mg/kg/day	Treated 100 mg/kg/day
Microsomal protein (mg/g liver)	34.1 ± 1.3	33.0 ± 2.0	34.9 ± 2.1
Cytochrome P450 (nmoles/g liver)	11.2 ± 0.5	10.3 ± 0.7	8.7 ± 0.4†
Aniline hydroxylase (nmoles product/g liver/hr)	319 ± 24	320 ± 17	296 ± 20
Ethylmorphine *N*-demethylase (nmoles product/g liver/hr)	850 ± 181	947 ± 406	588 ± 127

* Values are expressed as mean ± S.E.M.: there were eight animals/group.
† Significance levels ('*t*' test or analysis of variance) test vs control $P < 0.01$.

Table 2. Effect of repeated oral administration of isosorbide dinitrate on hepatic cytosol drug-metabolising enzyme activity

Parameter*	Controls	Treated 10 mg/kg/day	Treated 100 mg/kg/day
Soluble protein (mg/g liver)	69.8 ± 0.8	62.5 ± 1.8‡	65.0 ± 2.7
1,2-Dichloro-4-nitrobenzene conjugation† (μmoles conjugate formed/g liver/hr)	31.4 ± 1.1	29.1 ± 1.6	32.0 ± 1.4
Benzylideneacetone conjugation† (μmoles thiol reacted/g liver/hr)	215 ± 12	186 ± 11	202 ± 15
Glyceryl trinitrate denitration† (μmoles nitrite formed/g liver/hr)	10.0 ± 0.7	8.0 ± 0.8‡	10.1 ± 0.5

* Values are expressed as mean ± S.E.M.; there were eight animals/group.
† Glutathione S-transferase activity.
‡ Significance level ('*t*' test or analysis of variance), test vs control, $P < 0.05$.

not significantly, than that of controls (Table 1). Glutathione S-transferase activity towards glyceryl trinitrate, in the hepatic cytosol of rats dosed at 10 mg/kg/day, was significantly lower ($P < 0.05$) than controls (Table 2), although if enzyme activity is expressed in terms of soluble protein concentrations, no significant treatment-related differences were detected. The significant decrease in glutathione S-transferase activity towards glyceryl trinitrate in treated rats reflected the lower soluble protein concentrations in these animals.

Of the various rat liver glutathione S-transferases known [5], "transferase B" (ligandin) accounts for about 5 per cent of hepatic soluble protein [19] and possibly this S-transferase was present in lower concentrations in the livers of rats dosed with isosorbide dinitrate at 10 mg/kg/day. The work of Habig *et al.* [6] indicates that this S-transferase has relatively more activity towards glyceryl trinitrate than towards benzylideneacetone or 1,2-dichloro-4-nitrobenzene. Thus measurement of glutathione S-transferase activity towards glyceryl trinitrate is more likely to reveal changes in the activity or concentrations of "transferase B" than measurements involving the other two substrates used. Hepatic rat "transferase B" has been shown to be induced about two-fold by phenobarbitone treatment of animals [20, 21] as is glyceryl trinitrate metabolism *in vitro* [22, 23].

Repeated exposure of humans and animals, such as rats, to organic nitrates is claimed to lead to a decreased responsiveness to the pharmacological effects of the drug [24]. A possible explanation for this phenomenon is enhancement of drug biotransformation caused by induction of the organic nitrate metabolising enzymes, the GSH S-transferases. These studies (Tables 1 and 2) show that isosorbide dinitrate is probably not an inducer of GSH S-transferases or of microsomal drug-metabolising enzymes in rats at dosages up to 100 mg/kg.

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REFERENCES

1. P. Needleman and F. Hunter, Jr., *Molec. Pharmac.* **1**, 77 (1965).
2. P. Needleman and J. C. Krantz, Jr., *Biochem. Pharmac.* **14**, 1225 (1965).
3. L. A. Heppel and R. J. Hilmoe, *J. biol Chem.* **183**, 129 (1950).
4. P. Needleman, S. Lang and E. M. Johnson, Jr., *J. Pharmac. exp. Ther.* **181**, 489 (1972).
5. W. B. Jakoby, W. H. Habig, J. H. Keen, J. N. Ketley and M. J. Pabst, in *Glutathione: Metabolism and Function* (Eds I. M. Arias and W. B. Jakoby) p. 189. Raven Press, NY (1976).
6. W. H. Habig, J. Keen and W. B. Jakoby, *Biochem. biophys. Res. Commun.* **64**, 501 (1975).
7. J. H. Keen, W. H. Habig and W. B. Jakoby, *J. biol. Chem.* **251**, 6183 (1976).

8. A. H. Conney, *Pharmac. Rev.* **19**, 317 (1967).
9. H. Remmer, *Eur. J. clin. Pharmac.* **5**, 116 (1972).
10. J. O. Hunter and L. F. Chasseaud, in *Progress in Drug Metabolism* (Eds J. W. Bridges and L. F. Chasseaud) p. 129. Wiley, London (1976).
11. E. Boyland and L. F. Chasseaud, *Biochem. J.* **115**, 985 (1969).
12. T. Omura and R. Sato, *Meth. Enzym.* **10**, 556 (1967).
13. E. D. Wills, *Biochem. J.* **113**, 333 (1969).
14. J. Cochin and J. Axelrod, *J. Pharmac. exp. Ther.* **125**, 105 (1959).
15. J. Booth, E. Boyland and P. Sims, *Biochem. J.* **79**, 516 (1961).
16. L. F. Chasseaud, *Biochem. J.* **131**, 765 (1973).
17. S. Al-Kassab, E. Boyland and K. Williams, *Biochem. J.* **87**, 4 (1963).
18. O. H. Lowry, N. J. Rosebrough, A. L. Fair and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
19. G. Fleischner, J. Robbins and I. M. Arias, *J. clin. Invest.* **51**, 677 (1972).
20. W. H. Habig, M. J. Pabst, G. Fleischner, Z. Gatmaitan, I. M. Arias and W. B. Jakoby, *Proc. natn. Acad. Sci. U.S.A.* **71**, 3879 (1974).
21. N. Kaplowitz, J. Kuhlenkamp and G. Clifton, *Biochem. J.* **146**, 351 (1975).
22. P. Needleman and A. B. Harkey, *Biochem. Pharmac.* **20**, 1867 (1971).
23. N. H. Lee and F. M. Belpaire, *Biochem. Pharmac.* **21**, 3171 (1972).
24. P. Needleman and E. M. Johnson, Jr., in *Organic Nitrates* (Ed. P. Needleman) p. 97. Springer-Verlag, Berlin (1975).

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Alteration in serum tyrosine and tryptophan concentrations associated with the induction of physical dependence on ethanol in mice

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Changes in central monoamine neurotransmitter metabolism have frequently been reported in association with the development of physical dependence on ethanol in laboratory animals [1–3]. During the physical syndrome of withdrawal from ethanol further changes in central metabolism of monoamines occur which play important roles in behavioural aspects of the withdrawal syndrome [4, 5, 1]. The reasons for changes in central monoamine metabolism during the induction of ethanol dependence are therefore of interest.

There is some evidence that concentrations of the amino acid precursors for monoamine biosynthesis in the brain are altered in ethanol-dependent animals [3, 6] and it has been suggested that altered peripheral handling of these precursors, notably by the liver, may contribute to changes in central amine metabolism induced by ethanol [7, 8]. We now report that the induction of ethanol dependence in mice is associated with alterations in concentrations of tyrosine and free and bound tryptophan in plasma. Such changes may influence central amine metabolism [9].

MATERIALS AND METHODS

Male T.O. Swiss strain mice of 20–25 g obtained from LAB Centre, Dagenham, Essex, were made physically dependent on ethanol in the way previously described [1]. This method involves administration of ethanol by inhalation in increasing concentration to groups of mice. After administration of ethanol by this regime for 5 days, there is little sign of physical dependence, whereas after 10 days all mice show signs of a physical syndrome (cf. [1]) when ethanol is removed. The withdrawal syndrome can be observed for 10–12 h in most mice, and is at its peak after 4–5 h.

Mice were killed by decapitation after administration of ethanol for 5 and 10 days or after 5 and 10 h of the ethanol withdrawal syndrome. Mixed arterial and venous blood was obtained from the neck and collected into heparinised glass vials. After centrifugation at 10,000 g for 30 sec. Serum was removed for estimation of amino acids. Serum free tyrosine was estimated by the method of Waalkes and Udenfriend [10], serum total tryptophan by the method of Denckla and Dewey [11]. Free tryptophan was separated from the protein-bound fraction by the method of Knott and Curzon [12]. Serum from the pooled blood of two mice was used for free tryptophan estimation. In all cases comparison between values obtained was made with those from control animals exposed to the same environmental conditions, but with the absence of ethanol in inspired air.

RESULTS

As shown in Table 1, administration of ethanol for 5 days causes no significant change in concentrations of tyrosine in serum, but significantly reduces serum total tryptophan. The administration of ethanol for 10 days, a period sufficient to induce physical dependence, is associated with a significant increase in serum tyrosine and in serum total tryptophan concentrations. Both were increased to about 50 per cent above control levels. When separation of total tryptophan into the free and bound fractions was achieved, it was observed that, in ethanol dependent mice, the increase in total tryptophan was reflected in a smaller, but significant ($P < 0.05$) increase in free tryptophan. Control animals showed free tryptophan concentrations in serum of 4.78 ± 0.28 μg/ml ($n = 5$) whereas the value for ethanol dependent mice was 5.92 ± 0.33 μg/ml ($n = 5$).

During the syndrome of withdrawal from ethanol concentrations of tyrosine in serum remained significantly elevated above control (approximately double control values when the withdrawal syndrome was at its height). Con-