Biochemical Pharmacology, Vol. 28, pp. 1139–1141.

0006-2952/79/0401-1139 $02.00/0

THE EFFECT OF PROLONGED ETHANOL INTAKE ON SOME CARCINOGEN-ACTIVATING ENZYMES IN MICE

IFOR D. CAPEL, HELEN M. DORREL, MARILYN JENNER, MARISA H. PINNOCK and DONALD C. WILLIAMS

Research Department, Marie Curie Memorial Foundation, The Chart, Oxted, Surrey, RH8 0TL, England

(*Received* 23 *June* 1978; *accepted* 5 *October* 1978)

Abstract—Cytochrome P-450 and aryl hydrocarbon (benzo(a)pyrene) hydroxylase levels were increased in mice which received 1 or 10 per cent v/v absolute ethanol in their drinking water for periods of 24 or 32 weeks. These increases corresponded to a consistent rise in microsomal protein values throughout the experiment. The binding of benzo(a)pyrene to DNA was significantly increased during 1 per cent ethanol treatment, but had returned to the control level after 20 weeks. In the mice receiving 10 per cent ethanol, the binding of benzo(a)pyrene was consistently decreased, achieving the control level only after 32 weeks.

Reported epidemiological associations between excessive alcohol intake and the incidence of certain types of cancer [1–3] have stimulated the necessity to elucidate the interaction between alcohol and the carcinogen-metabolising enzymes [4]. Recent reviews imply that contradictory results have been obtained previously because the metabolic effects of alcohol differ according to the amount administered, duration of exposure, and the dosing regimen. With long term ethanol ingestion, however, it seems probable that the cytochrome P-450-linked microsomal ethanol oxidising system (MEOS) is responsible for the greater part of the observed adaptive increase in ethanol oxidation [5, 6]. In many instances the increases in the MEOS have been paralleled by an increase in the activity of other mixed function oxidase enzymes responsible for drug metabolism [6–8]. In this experiment the effect on the hepatic microsomes of mice of prolonged exposure to ethanol at a sub-chronic and chronic level are investigated and changes in some of the parameters associated with "carcinogen-activation" monitored.

MATERIALS AND METHODS

Chemicals. 2.5 l portions of absolute ethanol were redistilled at 78° discarding the first and last 250 ml portions of each distillate. [G-^{3}H] Benzo(a)pyrene, which was 98 per cent pure and further purified by t.l.c. to a constant specific activity of 27.5 Ci/m-mole, was purchased from the Radiochemical Centre, Amersham, Bucks. Instagel® was purchased from Packard Inc., Caversham, Bucks. All other chemicals were of the purest grade available and obtained from either Sigma or BDH, both of Poole, Dorset.

Animals and dosage. Male albino TO (Theiller-Original) mice of body weight 25 ± 2 g were obtained from Tuck and Son, Battlebridge, Essex and maintained on PRM diet purchased from Dixons, Ware, Herts. The drinking water of the mice, which was renewed daily, contained either 1 or 10 per cent (v/v) redistilled alcohol. Each group of mice consumed, on average, a constant 80–100 ml of the aqueous alcohol solution per day throughout the experiment. The groups of 20

Table 1. The effect of oral intake of ethanol (1% v/v) on the livers of mice

Duration of treatment (weeks)	Average body weight (g)	Liver weight (g)	Microsomal protein mg/g	Cytochrome P-450 (a)	Aryl hydrocarbon hydroxylase (b)	(c)	DNA binding of benzo(a)pyrene (d)	(e)
2	32.3 ± 0.37	2.1 ± 0.052	20.9 ± 0.44	1.5 ± 0.04	0.24 ± 0.010	4.9	2.6 ± 0.2	46
4	35.2 ± 0.53	2.2 ± 0.068	20.3 ± 0.58	1.6 ± 0.09	0.32 ± 0.008	6.5	7.3 ± 1.0	130
6	36.3 ± 0.62	2.1 ± 0.050	19.2 ± 0.60	1.6 ± 0.06	0.47 ± 0.032	9.0	13.5 + 0.5	240
8	39.4 ± 0.46	2.1 ± 0.041	24.4 ± 0.72	1.8 ± 0.06	0.40 ± 0.022	9.7	9.7 ± 1.3	172
12	40.3 ± 0.59	2.5 ± 0.058	21.8 ± 0.78	1.8 ± 0.08	0.34 ± 0.014	7.3	12.5 ± 1.1	222
16	42.4 ± 0.62	2.3 ± 0.059	26.6 ± 0.60	1.3 ± 0.03	0.28 ± 0.008	7.4	7.5 ± 0.7	133
20	42.9 ± 0.69	2.4 ± 0.050	27.3 ± 0.82	1.4 ± 0.05	0.36 ± 0.006	9.8	5.6 ± 0.6	99
24	44.9 ± 0.64	2.4 ± 0.060	30.2 ± 1.11	1.3 ± 0.04	0.23 ± 0.022	7.0	4.4 ± 0.9	79
Control*	36.2 ± 6.8	2.3 ± 0.05	23.0 ± 2.13	1.3 ± 0.09	0.24 ± 0.13	5.5	5.6 ± 0.6	100

* Control values are the means and S.D. of results obtained with groups of 5 animals sacrificed at each of the intervals indicated in Table 2.

(a) Expressed as nmoles/mg of microsomal protein. (b) Expressed as nmoles/mg of microsomal protein/min. (c) Expressed as nmoles/g liver. (d) Expressed as d.p.m. × 1000 [^{3}H]benzo(a)pyrene bound per mg DNA. (e) Expressed as a percentage of the control value.

Table 2. The effect of oral intake of ethanol (10% v/v) on the livers of mice

Duration of treatment (weeks)	Average body weight (g)	Liver weight (g)	Microsomal protein (mg/g)	Cytochrome P-450 (a)	Aryl hydrocarbon hydroxylase (b)	(c)	DNA binding of benzo(a)pyrene (d)	(e)
4	37.6 ± 0.74	2.42 ± 0.044	19.4 ± 0.54	1.7 ± 0.06	0.11 ± 0.013	2.1	3.0 ± 0.5	53
8	41.0 ± 0.82	2.58 ± 0.056	23.3 ± 0.63	1.4 ± 0.08	0.11 ± 0.028	2.6	1.6 ± 0.4	29
12	40.8 ± 0.69	2.47 ± 0.058	20.0 ± 0.44	1.5 ± 0.05	0.15 ± 0.030	3.0	3.6 ± 0.3	64
16	41.4 ± 0.89	2.56 ± 0.076	29.3 ± 2.24	1.3 ± 0.05	0.14 ± 0.056	4.1	4.9 + 0.7	87
20	41.4 ± 0.62	2.38 ± 0.048	33.3 ± 2.65	1.2 ± 0.05	0.19 ± 0.017	6.3	4.4 ± 0.1	78
24	41.1 ± 0.75	2.54 ± 0.074	32.5 ± 1.88	1.4 ± 0.08	0.14 ± 0.003	4.7	4.0 ± 0.4	72
28	41.8 ± 0.51	2.35 ± 0.074	37.4 ± 0.65	1.3 ± 0.08	0.22 ± 0.002	8.3	4.7 ± 0.5	84
32	40.6 ± 0.70	2.11 ± 0.053	37.5 ± 2.12	1.3 ± 0.06	0.27 ± 0.005	9.3	5.8 ± 0.4	103
Control*	36.2 ± 6.8	2.3 ± 0.05	23.0 ± 2.13	1.3 ± 0.07	0.24 ± 0.13	5.5	5.6 ± 0.6	100

* Control values are the means and S.D. of results obtained with groups of 5 animals sacrificed at each of the intervals indicated above.
(a) Expressed as nmoles/mg microsomal protein. (b) Expressed as nmoles/mg microsomal protein/min. (c) Expressed as nmoles/g liver. (d) Expressed as d.p.m. × 1000 [^{3}H]benzo(a)pyrene bound per mg DNA. (e) Expressed as a percentage of the control value.

ethanol-treated mice and 5 control animals (maintained on ethanol-free water) were sacrificed by cervical dislocation at the time intervals indicated in Tables 1 and 2 and their livers excised and chilled on ice.

Incubations and assays. The livers were homogenised in sucrose containing Tris pH 7.4 buffer and the microsomes sedimented by centrifugation. The microsomes were washed and then the protein determined by the method of Lowry *et al.* [9], cytochrome P-450 by the method of Omura and Sato [10] and aryl hydrocarbon (benzo(a)pyrene) hydroxylase by a radiometric technique [11].

To determine the DNA binding of benzo(a)pyrene each incubate contained: 20 mM sodium citrate pH 7.4 buffer (4.06 ml), 3.0 mg of calf thymus DNA in buffer (1.0 ml), 4.0 mg of microsomal suspension in 1.15 per cent KCl (0.5 ml), a NADPH-generating system comprising 14.1 mg of glucose 6-phosphate, 4.6 mg NADP and 2 units of glucose 6-phosphate dehydrogenase all in buffer (0.3 ml) and 0.1 M $MgCl_2$ solution (0.1 ml). To this incubation mixture was added 40 μl of a solution containing 2.5 mCi/mg/ml of benzo(a)pyrene in acetone. The mixture was incubated for 30 min at 37°, after which time the DNA was isolated and estimated. The amount of bound radioactivity was determined essentially as described by Grover and Sims [12].

Radiochemical analyses. Duplicate analyses were performed on each sample which was esimated in Insta-gel®. Samples were counted for 2 cycles, each of 10 min duration, in a Packard 4250 liquid scintillation spectrometer. Counting efficiency was determined by external standardisation.

RESULTS

The effect of prolonged intake of ethanol at 1 and 10 per cent on the livers of mice is given in Tables 1 and 2 respectively. With control animals there was no significant change in the levels of either the microsomal protein, cytochrome P-450, aryl hydrocarbon (benzo[a]pyrene) hydroxylase or DNA binding of benzo(a)pyrene during 24 weeks. Following both 1 and 10 per cent ethanol intake the microsomal protein level increased progressively to approximately 1/3 higher than that of the the control value at 24 weeks, and to 2/3 higher than the controls after 34 weeks ingestion of 10 per cent ethanol.

Alcohol intake increased the cytochrome P-450 level to a maximum of 140 per cent of the control value, the level appearing normal after 16 weeks, but the total liver content was elevated as a result of the increase in microsomal protein. Similarly, after 1 per cent ethanol ingestion, the aryl hydrocarbon (benzo[a]pyrene) hydroxylase level was increased initially, and the increase in microsomal protein resulted in an average increase of 50 per cent in the liver enzyme content. With the 10 per cent ethanol treated animals, there was a decrease in aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity during the first 16 weeks of intake, the level "recovering" after 4 weeks to achieve a maximum of 170 per cent of the control value after 32 weeks.

The DNA binding of benzo(a)pyrene was increased 2-fold during the period 6–12 weeks of 1 per cent ethanol ingestion, but had fallen below the control value before the termination of the experiment. The carcinogen binding level of the 10 per cent treated animals was apparently lowered and achieved the control level only after 32 weeks.

DISCUSSION

Except for correlations between ethanol abuse and oral cancer [1–4], much of the epidemiological evidence linking alcohol with cancer is equivocal and the subject remains controversial [13]. The primary organ concerned with ethanol metabolism is the liver and the effect of alcohol abuse on hepatic metabolism is also controversial. A "hypermetabolic state" induced by ethanol has been postulated [14] but it is likely that the adaptive increase in the hepatic capacity to metabolise the drug is largely a result of the enhancement of the MEOS system and not due to increases in the mitochondrial alcohol dehydrogenase or catalase enzyme system [5, 6, 15]. This increase in MEOS activity could be paralleled by increases in other general mixed function oxidase enzymes, including those concerned with carcinogen metabolism, and there are conflicting reports of the effect of alcohol on microsomal en-

zymes [16–18]. Generally, any dose level of alcohol above 5 per cent v/v in water is regarded as producing liver damage [19]. It has been suggested by Cinti *et al.* [20] that low doses of ethanol stimulate, and higher doses inhibit microsomal enzymes, but little data on prolonged ethanol intake is available. It has also been demonstrated that the diet and dosing regimen are crucial to the effect of alcohol on microsomal enzymes [8, 19]. A higher dietary lipid content stimulates the enhancing effect of alcohol on microsomal enzyme activity [19, 21].

In the present experiment, ethanol increased the microsomal capacity to oxidise benzo(a)pyrene for the first few months of 1 per cent ingestion by increasing aryl hydrocarbon (benzo[a]pyrene) hydroxylase activity. Higher doses and longer duration ethanol intake resulted in an increase in microsomal protein content and presumably increased synthesis of aryl hydrocarbon (benzo[a]pyrene) hydroxylase. This is in agreement with reports that ethanol ingestion for shorter periods of time increases microsomal protein [17] and causes proliferation of the smooth endoplasmic reticulum [6, 22]. It is concluded that the level of aryl hydrocarbon (benzo[a]pyrene) hydroxylase is increased following intake of high as well as lower concentrations of ethanol but that the adaptive metabolic response to higher alcohol concentrations was slower. Thus, the degree to which alcohol stimulates or inhibits this microsomal enzyme varies not only with the amount but with the duration of exposure.

Determination of the binding of carcinogens to DNA has been reported to be the most reliable estimate of carcinogenicity [23]. The observed increase in the aryl hydrocarbon (benzo[a]pyrene) hydroxylase levels in the livers of male mice might be expected to increase DNA binding of this carcinogen. The binding of benzo(a)pyrene was indeed significantly raised when the hydroxylase activity was increased, as in the case of the 1 per cent treated animals, although with the higher doses of alcohol the binding was reduced. Recent reports implicate the aryl hydrocarbon hydroxylase located in the nuclear envelope as being responsible for the activation of benzo(a)pyrene to its proximate carcinogen [24] and presumably this enzyme would not be induced during the adaptive increase of the MEOS.

It is difficult to predict the *in vivo* effect of an alcohol induced MEOS adaptation in man from increases in murine cytochrome P-450 and aryl hydrocarbon (benzo[a]pyrene) hydroxylase. It has been shown that there is more than one species of cytochrome P-450 [25]. Recent reports implicate a distinct type of cytochrome in rats which is induced as part of the adaptive increase of the MEOS to alcohol intake [26]. Presumably this species of P-450 would not catalyze the P-448-mediated carcinogen activation reactions. Similarly, the presence of more than one aryl hydrocarbon hydroxylase enzyme has been demonstrated in the livers of rats, the relative content of each varying with age and sex [27].

This experiment demonstrated that an enzyme involved in carcinogen activation can be increased as a consequence of the adaption of the hepatic MEOS to prolonged ethanol ingestion. The relative importance of this increase must be measured against the actions of the other inducing agents, e.g. smoking [28], before its bearing on the adverse epidemiological observations can be assessed.

REFERENCES

1. E. L. Wynder, I. D. J. Bross and E. Day, *Cancer, N.Y.* **9**, 86 (1956).
2. A. A. Mihas and W. G. Doos, *Oncology* **31**, 280 (1975).
3. I. D. J. Bross and J. Coombs, *Oncology* **33**, 136 (1976).
4. E. L. Wynder, *J. Am. Dietetic Assoc.* **71**, 385 (1977).
5. A. I. Cederbaum, E. Dicker, C. S. Lieber and E. Rubin, *Biochem. Pharmac.* **27**, 7 (1977).
6. A. I. Cederbaum, E. Dicker, C. S. Lieber and E. Rubin, Alcoholism: *Clin. exp. Res.* **1**, 27 (1977).
7. F. Tobon and E. Mezey, *J. Lab. Clin. Med.* **77**, 110 (1971).
8. E. Rubin, P. Bacchin, H. Gang and C. S. Lieber, *Lab. Invest.* **22**, 569 (1970).
9. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
10. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
11. T. Hayakawa and S. Udenfriend, *Analyt. Biochem.* **51**, 501 (1973).
12. P. L. Grover and P. Sims, *Biochem. J.* **110**, 159 (1968).
13. R. R. Monson and J. L. Lyon, *Cancer, N.Y.* **36**, 1077 (1975).
14. J. Bernstein, L. Videla and Y. Israel, *Biochem. J.* **134**, 515 (1973).
15. R. Teschke, S. Matsuzaki, K. Ohnishi, L. M. DeCarli and C. S. Lieber, *Alcoholism: Clin. exp. Res.* **1**, 7 (1977).
16. G. Powis, *Biochem. J.* **148**, 269 (1975).
17. P. Luoma and M. Vorne, *Acta pharmac. tox.* **33**, 442 (1973).
18. R. R. Ramsey and H. J. Fallon, *Gastroenterology* **62**, 174 (1972).
19. J.-G. Joly and C. Hetu, *Biochem. Pharmac.* **24**, 1475 (1975).
20. D. L. Cinti, R. Grundin and S. Orrenius, *Biochem. J.* **134**, 367 (1973).
21. D. J. Tuma, M. F. Sorrell, J. A. Vanderhoof and A. J. Barak, *Proc. Soc. exp. Biol. Med.* **155**, 67 (1977).
22. E. Rubin, F. Hutterer and C. S. Lieber, *Science, N.Y.* **159**, 1469 (1968).
23. E. Huberman and L. Sachs, *Int. J. Cancer* **19**, 122 (1977).
24. K. Alexandrov, *Europ. J. Cancer* **13**, 847 (1977).
25. J. E. Tomaszewski, D. M. Jerina, W. Levin and A. H. Conney, *Archs Biochem. Biophys.* **176**, 788 (1976).
26. J.-P. Villeneuve, P. Mavier and J.-G. Joly, *Biochem. biophys. Res. Commun.* **70**, 723 (1976).
27. F. J. Wiebel and H. V. Gelboin, *Biochem. Pharmac.* **24**, 1511 (1975).
28. R. K. Abramson and J. J. Hutton, *Cancer Res.* **35**, 23 (1975).