J. Pharm. Pharmacol. 1993, 45: 81–85
Received May 9, 1992

The Urinary Excretion of Tryptophan and Tryptophan Metabolites in the Chronic Ethanol-fed Rat

ADRIAN B. BONNER, SARAH BRIEN AND VICTOR R. PREEDY*

*The Addictive Behaviour Centre, The Roehampton Institute, London SW15 3SN, UK, and *The Department of Clinical Biochemistry, King's College School of Medicine and Dentistry, London SE5 9PJ, UK*

Abstract—An investigation was made into the hypothesis that chronic ethanol ingestion disturbs the metabolism of tryptophan which is reflected by alterations in the urinary excretion of the metabolites 5-hydroxyindoleacetic acid (5-HIAA), anthranillic acid (AA) and indoleacetic acid (IAA). In particular, we investigated whether experimental chronic alcoholism is associated with a decrease in the tryptophan metabolite ratios as suggested in the literature. Male Wistar rats were chronically fed a nutritionally-complete liquid diet in which ethanol comprised 35% of total calories: controls were pair-fed identical amounts of the same diet in which ethanol was replaced by isocaloric glucose. At 6 weeks, 24 h urine samples were collected for the analysis of tryptophan, 5-HIAA, AA and IAA by HPLC. During ethanol-feeding there were reductions in the daily urinary excretion (i.e. μmol/24 h) of tryptophan (−57%, $P=0.026$) and concomitant increases in 5-HIAA excretion (62%, $P=0.057$). Expression of data in terms of lean tissue mass (i.e. urinary creatinine) revealed identical conclusions. An analysis was performed on the molar ratios of these urinary analytes. The tryptophan: total metabolite ratio was significantly decreased (by −53%), but the AA: total metabolite ratio was not significantly altered ($P=0.102$). The ratios 5-HIAA/AA and 5-HIAA/IAA were slightly increased, but they did not attain statistical significance ($P>0.351$). It was concluded that chronic ethanol feeding is associated with significant changes in the urinary excretion of tryptophan and its related metabolites. Many of the above changes were contrary to previous clinical data and this may be due to dietary and nutritional deficiencies or to alterations in the diurnal pattern of 5-hydroxytryptamine and tryptophan metabolism.

The neurotransmitter 5-hydroxytryptamine (5-HT) has been implicated in the hypothalamic control of ethanol intake (Myers & Melchior 1975). Decreased levels of 5-HT have been demonstrated in the brains of experimental animals with increased alcohol consumption (Myers & Melchior 1975). Biochemical studies on genetically selected animals, which have high or low ethanol intake, have also confirmed the involvement of 5-HT in modulating ethanol consumption (McBride et al 1990). It is possible that 5-HT deficiency in the brain could be a contributing factor in the development of alcoholism (Cloninger 1987). If this is the case two pharmacological strategies are available. The potential use of 5-HT_3-receptor antagonists has been discussed by Sellers (1992), secondly 5-HT neuronal activity could be facilitated using compounds that will release 5-HT, block 5-HT re-uptake, or act as selective 5-HT agonists. With regard to the latter, dietary tryptophan deficiency might exacerbate or contribute to this phenomenon. This is because tryptophan is the principle precursor of 5-HT and various studies have shown that dietary tryptophan deficiency leads to a reduction in brain 5-HT (Gal & Drewes 1962; Culley et al 1963; Fernstrom & Hirsch 1977).

From the above it follows that the monitoring of brain 5-HT metabolism may provide important information concerning the pathogenesis of alcohol dependence and toxicity. However, brain levels of 5-HT are difficult to investigate on a routine basis in animals and virtually impossible in man. Furthermore, the interpretation of such data would be complicated by the fact that studies have shown that cerebral 5-HT_{1A}-receptor density and sensitivity are also altered in experimental alcoholism (McBride et al 1991). However, urinary 5-hydroxyindoleacetic acid (5-HIAA), a product of 5-HT catabolism, has been used to monitor endogenous 5-HT levels (Olson et al 1960; Perman 1960; Akhter et al 1978; Thomson & McMillen 1987). 5-HIAA has been measured in conjunction with the other main urinary markers of tryptophan metabolism, namely anthranillic acid (AA) and indoleacetic acid (IAA). The rationale of determining these latter analytes is based on the assumption that the ratios between a tryptophan metabolite in one pathway will remain the same regardless of the amounts of the metabolites excreted or the concentration in the urine (Thomson & McMillen 1987). Although we feel this concept is an over-simplification, Thomson & McMillen (1987) have nevertheless suggested that chronic alcoholics have reduced ratios of the urinary metabolites 5-HIAA:AA and 5-HIAA:IAA. Thus, the use of these ratios removes some of the confounding influences of diet which are thought to be altered in alcoholism. This rationale is supported by various studies showing that alcoholics are likely to have impoverished diets as a result of their lifestyle and socio-economic status (reviewed by World et al 1985).

However, many clinical investigations into the role of 5-HT and tryptophan-related metabolites in chronic ethanol toxicity have not strictly taken nutritional factors into consideration. More importantly, innumerable studies have taken single urinary voids for analysis. It is possible that investigations on single urinary samples may be subject to errors due to altered diurnal patterns and artifactual changes due to occasional feeding. In addition some studies (albeit clinical) have assayed urinary metabolites during abstinence.

Correspondence: A. B. Bonner, The Addictive Behaviour Centre, The Roehampton Institute, London SW15 3SN, UK.

In this study we analysed the characteristics of experimental whole-body 5-HT and tryptophan metabolism during alcoholic intoxication in rats. A method was employed which ensured that both control and ethanol-fed rats received identical amounts of dietary tryptophan and the diets were also isovolumetric and isocaloric (Preedy et al 1988, 1990). To resolve problems inherent in the analysis of single urinary samples during episodic feeding, urine samples were collected over 24 h.

Materials and Methods

Animals

The experimental animal model employed here was devised by Lieber & DeCarli (1989) and recently described by Preedy et al (1988, 1990). Male rats were reared in a Home Office-approved (humidified and temperature controlled) animal house on a 12 h light/dark cycle commencing at 0730 h. Animals were individually housed in wire-bottomed cages and fed a solid laboratory diet for 3–7 days, until they attained the desired body weight of approximately 0·1 kg. They were then ranked in order of body weight. The rats were pair-matched such that the mean body weights of each group were approximately identical.

Ethanol feeding studies

On the first day of the study both control and treated groups were provided with free access to 120 mL of the control liquid diet (i.e. without ethanol). The control diet was freshly prepared each day and consisted of: 121 g of a commercial food-drink supplement (Vita Food, Boots Company plc, Nottingham, UK), 85 g glucose (Hospital Pharmacy), 10 g caesin (BDH Chemicals Ltd, Poole, Dorset, UK), a vitamin supplement (Oravite 7, Beecham Group, Brentford, Middlesex, UK) and 800 mL water. The final volume was 930 mL (Preedy et al 1990, 1991). This diet provided 19, 15 and 66% of total energy as fat, protein and carbohydrate, respectively, as well as minerals, vitamins and other nutrients. On the following days the diet freely available to the ethanol group of animals was prepared by substituting 42·5 g glucose with 28·3 mL ethanol.

Control rats were fed their respective diet on day 2. On the third day the volume consumed by each alcoholic rat was recorded and an identical volume of control diet given to its complementary pair. This procedure was repeated each day and any liquid diet remaining in the drinking bottles of control rats was added to the following day's feed (Preedy et al 1990). On day 5, and thereafter, the proportion of alcohol in the diet was increased to 35%; the ethanol content was 56·5 mL and the glucose content was zero. The composition of the other ingredients remained unaltered. The experiment was terminated after 6 weeks.

Rats were housed in Tecniplast metabolic cages (Labcare Precision Ltd, Aldington, Kent, UK) to facilitate collection of urine. On the penultimate day of the experiment urine was collected over a 24-h period from the ethanol-fed rats. On the following day, urine samples were collected from the control rats. Urine was stored at −70°C until analysis.

Instrumentation and analytical methods

Urinary samples were assayed by HPLC. The semi-automated Shimadzu system incorporated an auto-injector (Sil-6A), system controller (Scl-6A), pump (LC-6A) and an oven (CTO-6A) containing the 25 cm Spherisorb 5 μm analytical column, internal diameter 4·6 mm, with integral 1 cm guard column. A Shimadzu electrochemical detector (LECD-6A) was linked to an integrator (CR4A). The buffer was prepared by mixing together 350 mL 0·1 M sodium phosphate (monobasic), 5 mL 0·1 M acetic acid, 11 mg Na_2EDTA and 18·6 mL methanol. The pH of the buffer was adjusted to 4·35 with 1 M KOH. The solution was filtered through a Millipore GS 0·22 μm filter and de-gassed at the beginning of each day by helium. The buffer was pumped through the system at a flow rate of 1·8 mL min^{-1}. At a column temperature of 45°C the average system pressure was 2500 psi. The electrochemical detector was set at an oxidizing potential of +0·99 V. The volume of the standards and samples that were injected into the system was 100 μL and the average time of each analysis was 20 min.

Sample collection and analysis

Samples were collected in plastic tubes and stored at −70°C until use. After thawing, the samples were centrifuged at 12000 rev min^{-1} (MSE Micro Centaur) for 5 min and then diluted by adding 200 μL supernatant to 600 μL distilled water. This solution was added to disposable plastic mini-Eppendorf tubes which were loaded into the carousel of the auto-injector. Quantification of the data was performed using an absolute calibration method (areas). Standard solutions containing 5-HIAA, AA, IAA (generally 0·5 μM) and tryptophan (1 μM) were prepared and chromatographed in order to set up an identification file, to be used in the quantification of the samples. All standards were obtained from Sigma Chemicals (Poole, Dorset, UK) and were used to recalibrate the system after every fifth sample.

Each urine sample was assayed for creatinine by the method of Lustgaraten & Wenk (1972).

Statistics

All data are expressed as the mean and s.e.m. of six pairs of observations in each group. Differences between means were assessed by Student's *t*-test for paired samples. Significance was assumed to have occurred when P was equal to or less than 0·05.

Results

At the end of 6 weeks there were differences in mean final body weight (control 201·0 ± 6·2 g, ethanol-fed 183·0 ± 7·3 g, $P=0·003$). Mean urinary creatinine concentrations were reduced from 1·56 (controls) to 1·31 μmol L^{-1} (ethanol-fed, $P=0·097$). There was a difference in mean urinary volumes (control 31·8 mL, ethanol-fed 28·2 mL, $P=0·003$). These results are similar to those reported previously (Preedy et al 1991).

Table 1 shows urinary tryptophan, 5-HIAA, AA and IAA in control and ethanol-fed rats.

In Table 2 the 5-HIAA excretion has been presented as ratios of AA and IAA, as described by Thomson & McMillen (1987); these changes did not achieve statistical significance ($P>0·244$).

Table 3 shows individual metabolite concentrations as a

Table 1. Daily output of urinary metabolites.

	Tryptophan	5-HIAA	AA	IAA
Expressed as μmol/24 h				
Control	176·8 (37·0)	42·9 (13·3)	18·3 (9·6)	10·0 (1·3)
Ethanol-fed	77·0 (28·0)	69·4 (8·5)	11·2 (6·2)	14·1 (7·9)
P	0·026*	0·057	0·097	0·100
Difference from control	−57%	+62%	−39%	+41%
Expressed as μmol kg^{-1}/24 h				
Control	888·2 (187·5)	206·5 (62·5)	87·1 (44·5)	49·2 (5·4)
Ethanol-fed	426·5 (159·4)	386·7 (44·0)	59·5 (31·8)	78·1 (15·6)
P	0·038*	0·034*	0·127	0·053
Difference from control	−52%	+87%	−32%	+59%
Expressed as μmol (μmol creatinine)$^{-1}$				
Control	117·8 (25·0)	27·8 (8·5)	11·8 (5·8)	6·6 (1·0)
Ethanol-fed	66·9 (29·8)	54·4 (7·4)	8·7 (4·7)	11·8 (3·2)
P	0·104	0·008**	0·090	0·078
Difference from control	−43%	+96%	−27%	+78%

All data are the mean (s.e.m. in parenthesis) of 6 pairs of observations. Differences between means were assessed by Student's *t*-test for paired samples. *$P<0·05$, **$P<0·01$.

Table 2. Metabolite molar ratios in control and treated animals.

	5-HIAA:AA	5-HIAA:IAA
Control	46·62 (36·31)	4·21 (1·34)
Ethanol-fed	71·18 (47·55)	6·02 (1·49)
P	0·351	0·244
Difference from control	+53%	+43%

Data are mean (s.e.m. in parenthesis) of 6 pairs of observations. Differences between means were assessed by Student's *t*-test for paired samples after log transformation of the data. None of the differences between the means were statistically significant. ($P>0·05$).

Table 3. Tryptophan and other catabolites as a proportion of total metabolites.

	Tryptophan	5-HIAA	AA	IAA
Control	0·721 (0·082)	0·083 (0·037)	0·078 (0·042)	0·050 (0·011)
Ethanol-fed	0·335 (0·093)	0·482 (0·617)	0·080 (0·046)	0·093 (0·015)
P	0·009**	0·020*	0·102	0·061
Difference from control	−53%	+484%	+1%	+86%

Molar ratios are presented as the means (s.e.m. in parenthesis) of 6 pairs of observations. Differences between means were assessed by Student's *t*-test for paired samples after log transformation of the data. *$P<0·05$, **$P<0·01$.

proportion of the total metabolites (i.e. sum of tryptophan, 5-HIAA, AA and IAA). The tryptophan: total metabolite ratio was significantly decreased (by 53%, $P=0·009$) but there was no change in the AA: total metabolite ratio (+1%; $P=0·f02$).

Discussion

Dietary tryptophan can be directly incorporated into tissue protein or converted either to 5-hydroxytryptophan, tryptamine, *N*-formylkynurenine or other intermediary compounds. End-products of these pathways are 5-HIAA, IAA and AA, respectively, and are excreted in the urine. It follows that their urinary concentrations may provide suitable indices of circulating 5-HT, tryptamine or *N*-formylkynurenine turnover. However, any clinical study on the excretion of urinary substances has to consider the possible dietary contribution of those substances being studied (either the metabolites themselves or their precursors), and possible diurnal influences (which may or may not be altered by the patterns of feeding or sleeping). For example, alcoholics may have marked perturbations in nutritional intake, compared with abstinent counterparts (World et al 1985), and there may also be alterations in diurnal patterns of feeding and

sleeping which also affect 5-HT and AA concentrations (Kuhn et al 1968; Luce 1970). The urinary concentration of many tryptophan-related metabolites is increased by common dietary substances (Crout & Sjoerdsma 1959; Garb 1971; Foy & Parratt 1961). Thus, we feel that in many clinical studies, the effects of ethanol itself may not be unequivocally demonstrated.

To evaluate the effect of chronic ethanol consumption on the excretion of urinary metabolites we used a well-validated model, as recently described by Preedy et al (1988, 1989, 1990, 1991). The collection of urine over a 24-h period is of considerable importance in re-affirming the validity of our measurements. This is because rats fed the Lieber-DeCarli diet are anorexic and the counter-matched pair-fed controls are semi-starved (Preedy et al 1988, 1989). When presented with their food, there is episodic engorgement of the liquid diet, with ensuing episodic urination (Preedy et al 1989). Thus, spot urinary samples are invalid, but samples collected over 24 h are more appropriate for investigating the chronic effects of ethanol (Preedy et al 1989, 1991) and reflect perturbations due to ethanol itself.

In our study, there was a significant increase in 5-HIAA production in alcohol-fed rats, which contrasts with previous findings where 5-HIAA has been shown to decrease in urine of alcoholics (Olson et al 1960; Banki 1981). An explanation for this difference may be related to nutrition or increased availability of co-factors important in monoamine oxidase (MAO) metabolism. However, the latter is less likely, since gastrointestinal absorption of vitamins and nutrients, which are important in MAO metabolism, has been shown to have been impaired in such cases (World et al 1985). Alternatively, it is possible that ethanol stimulates the synthesis of (MAO), but this seems unlikely as chronic ethanol ingestion has been shown to have no overt effect on brain MAO (Major et al 1985; Morinan 1987). A more likely explanation is that tryptophan availability is increased due to the inhibition of the liver enzyme tryptophan pyrrolase which is the most important enzyme involved in tryptophan degradation (Badawy 1988). This suggestion is supported by the decrease of urinary AA, presumably controlled by tryptophan pyrrolase. Paradoxically, however, urinary tryptophan is decreased. We do not know the reason for this.

Reductions in the concentrations of urinary tryptophan may have reflected plasma levels which may in turn have influenced the synthesis of the neurotransmitter 5-HT in the brain. Plasma levels of tryptophan and its transport into the brain involve complex interrelationships with large neutral amino acids and non-esterified fatty acids (Fernstrom 1988). Thus physiological and biochemical factors which influence tryptophan concentrations in the peripheral circulation are subsequently important in controlling 5-HT-ergic levels within the brain. We did not examine plasma tryptophan levels and other amino acids in relation to fatty acids because this would have reflected episodic feeding patterns (Preedy et al 1989, 1991). Nevertheless, the overall data suggest that tryptophan metabolism is disturbed in ethanol-fed rats. A major contributing factor for the increased tryptophan availability may be the ethanol-induced reductions in tissue protein synthesis (Preedy et al 1990): this will increase tryptophan availability in the circulation.

Levels of IAA were also increased but no significant change was found in AA. It has been argued (Thomson & McMillen 1987) that reductions in the urinary 5-HIAA:AA (and also 5-HIAA:IAA) ratios might provide a means of identifying potential alcohol abusers as these ratios reflect an impairment in the 5-HT metabolic pathway. Presumably this would be due to lowered MAO activity or to changes in tryptophan pyrrolase activities. Our negative results pertaining to these ratios might be explained by the fact that ethanol had no effect on the activity of MAO or the liver enzyme tryptophan pyrrolase. In contrast, our data suggest that tryptophan pyrrolase may be decreased (to account for the suggestive fall in AA excretion). Alternatively, it is possible that urinary AA excretion does not reflect the activity of the kynurenine pathway which encompasses the conversion of tryptophan to kynurenine and to nicotinamide. However, in the study of Thomson & McMillen (1987) subjects were abstinent during measurements and only spot urinary samples were analysed from subjects which did not appear to have matched nutritional intakes; such differences in results may be methodological in origin. It would have been advantageous to assay kynurenine itself but there are many practical problems in its determination. A more promising approach for assessing the effects of 5-HT-ergic impairment due to chronic ethanol intake, which also takes into account the nutritional status, appears to be the use of molar ratios of tryptophan and 5-HIAA to the total metabolites (Table 3). It remains to be seen whether differences in these molar ratios also occur in the clinical situation.

Acknowledgements

We are exceedingly grateful for the support, interest and encouragement of Professor Timothy J. Peters. The helpful advice of Dr Roy Sherwood is also acknowledged.

References

Akhter, M. I., Clark, P. M. S., Kricka, L. J., Nicholson, G. (1978) Urinary metabolites of tryptophan, serotonin and norepinephrine. J. Stud. Alcohol. 39: 833–841

Badawy, A. (1988) Effects of alcohol on tryptophan metabolism. Biochem. Soc. Trans. 16: 254–256

Banki, C. M. (1981) Factors influencing monoamine metabolites and tryptophan in patients with alcohol dependence. J. Neural Transm. 50: 89–101

Cloninger, C. R. (1987) Neurogenetic adaptive mechanisms in alcoholism. Science 236: 410–415

Crout, J. R., Sjoerdsma, A. (1959) The clinical and laboratory significance of serotonin and catecholamines in bananas. New Eng. J. Med. 261: 27–30

Culley, W. J., Saunders, R. N., Mertz, E. T., Jolly, E. T. (1963) Effect of a tryptophan-deficient diet on brain serotonin and plasma tryptophan levels. Proc. Soc. Exp. Biol. 113: 645–648

Foy, J. M., Parratt, J. R. (1961) 5-Hydroxytryptamine in pineapples. J. Pharm. Pharmacol. 13: 382–383

Fernstrom, J. D. (1988) Tryptophan availability and serotonin synthesis in brain. In: Huether, G. (ed.) Amino Acid Availability and Brain Function in Health and Disease. NATO ASI Series vol H20, Springer-Verlag, Berlin, pp 137–146

Fernstrom, J. D., Hirsch, M. J. (1977) Brain serotonin synthesis: reduction in corn malnourished rats. J. Neurochem. 28: 877–879

Gal, E. M., Drewes, P. A. (1962) Studies on the metabolism of 5-hydroxytryptamine (serotonin) II. Effect of tryptophan deficiency in rat. Proc. Soc. Exp. Biol. 110: 368–371

Garb, S. (1971) Clinical Guide to Undesirable Drug Reactions and Interferences. Springer Publishing Co, New York, NY

Kuhn, E., Rysanek, K., Brodan, V. (1968) Alterations of tryptophan metabolism induced by sleep deprivation. Experientia 24: 901–902

Lieber, C. S., DeCarli, L. M. (1989) Liquid diet technique of ethanol administration: 1989 update. Alcohol Alcohol. 24: 197–211

Luce, G. G. (1970) Biological Rhythms in Psychiatry and Medicine. PHS Publication No. 2088, Washington, DC

Lustgaraten, J. A., Wenk, R. F. (1972) Simple, rapid, kinetic method for serum creatinine measurement. Clin. Chem. 18: 1419–1422

Major, L. F., Hawley, R. J., Saini, N., Garrick, N. A., Murphy, D. L. (1985) Brain and liver monoamine oxidase type A and type B activity in alcoholics and controls. Alcoholism 9: 6–9

McBride, W. J., Murphy, J. M., Lumeng, L., Li, T.-K. (1990) Serotonin, dopamine and GABA involvement in alcohol drinking of selectively bred rats. Alcohol 7: 199–205

McBride, W. J., Murphy, J. M., Gatto, G. J., Levy, A. D., Lumeng, L., Li, T.-K. (1991) Serotonin and dopamine systems regulating alcohol intake. Alcohol Alcohol. 26 (Suppl. 1): 411–416

Morinan, A. (1987) Reduction in striatal 5-hydroxytryptamine turnover following chronic administration of ethanol to rats. Alcohol Alcohol. 22: 53–60

Myers, R. D., Melchior, C. L. (1975) Alcohol drinking in the rat after destruction of serotonergic and catecholaminergic neurons in the brain. Res. Commun. Chem. Path. Pharmacol. 10: 363–378

Olson, R.E., Gursey, D., Vester, J.W. (1960) Evidence for a defect in tryptophan metabolism in chronic alcoholism. New Engl. J. Med. 263: 1169–1174

Perman, E. S. (1960) Observations on the effect of ethanol on the urinary excretion of histamine, 5-hydroxyindolacetic acid, catecholamines and 17-hydroxycorticosteroids in man. Acta Physiol. Scand. 51: 62–67

Preedy, V. R., Duane, P., Peters, T. J. (1988) Biological effects of chronic ethanol consumption. A reappraisal of the Lieber-De Carli liquid diet model with reference to skeletal muscle. Alcohol Alcohol. 23: 151–154

Preedy, V. R., Hammond, B., Iles, R. A., Davies, S. E. C., Gandy, J. D., Chalmers, R. A., Peters, T. J. (1991) Urinary excretion of nitrogenous and non-nitrogenous compounds in the chronic ethanol-fed rat. Clin. Sci. 80: 393–400

Preedy, V. R., Marway, J. S., Macpherson, A. J. S., Peters, T. J. (1990) Ethanol-induced smooth and skeletal muscle myopathy: use of animal studies. Drug Alcohol Depend. 26: 1–8

Preedy, V. R., Marway, J. S., Peters, T. J. (1989) Use of the Lieber-DeCarli liquid feeding regime with specific reference to the effects of ethanol on rat skeletal muscle RNA. Alcohol Alcohol. 24: 439–445

Sellers, E. M. (1992) 5-HT and alcohol abuse. Trends Pharm. Sci. 13: 69–71

Thomson, S. M., McMillen, B. A. (1987) Test for decreased serotonin/tryptophan metabolite ratios in abstinent alcoholics. Alcohol 4: 1–5

World, M. J., Ryle, P. R., Thomson, A. D. (1985) Alcoholic malnutrition and the small intestine. Alcohol Alcohol. 20: 89–124