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Neurochemical correlates of ethanol withdrawal: alterations in serotoninergic function

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Cessation of chronic ethanol administration, and elimination of ethanol from the body, results in a withdrawal syndrome in mice characterized by behavioural symptoms and hypothermia. During withdrawal, the accumulation rate of [^{14}C] 5-hydroxytryptamine (5-HT) from [^{14}C]tryptophan, was significantly lower in the brainstem of the ethanol-withdrawn animals than in controls. A similar pattern was seen in forebrain. When the rate of 5-HT accumulation was determined using pargyline, no differences occurred between control and ethanol-treated animals. The endogenous concentrations of tryptophan in plasma, and tryptophan, 5-HT and 5-hydroxyindoleacetic acid (5-HIAA) in brain were the same in ethanol-treated and control animals. It is suggested that the changes in accumulation of ^{14}C-5-HT and ^{14}C-5-HIAA in ethanol-withdrawn animals reflected alterations in electrical activity of serotoninergic neurons during withdrawal.

One of the characteristic features of the withdrawal syndrome noted in animals after chronic ethanol intake is an increased sensitivity to spontaneous (Goldstein, 1974; Hunt & Majchrowicz, 1974b), drug-induced (Tabakoff & Boggan, 1974) and sound-induced (Freund & Walker, 1971) seizures. Since Schlesinger, Boggan & Freedman (1968) and Lehmann (1967) demonstrated that lowering the functional concentrations of 5-HT in the cns predisposes rats and mice to both drug-induced and sound-induced seizures, we felt that an assessment of the parameters which influence 5-HT metabolism during the ethanol withdrawal syndrome was in order. Although several attempts have been made to monitor 5-HT turnover in animals chronically treated with ethanol during the time they were intoxicated (Kuriyama, Raucher & Sze, 1971; Yamanaka & Kono, 1974), to our knowledge only Hunt & Majchrowicz (1974a) and Frankel, Khanna & others (1974) have attempted to measure 5-HT turnover in brain after the animals had been withdrawn from chronic ethanol treatment. In those studies the monoamine oxidase inhibitor pargyline was used to assess 5-HT turnover; but pargyline alters the discharge pattern of brain 5-HT neurons (Aghajanian, Graham & Sheard, 1970). In addition, although the animals used by Frankel & others (1974) were tolerant to ethanol, no mention was made of whether they were physically dependent on ethanol. We have found that tolerance to and physical dependence on ethanol do not develop concomitantly in mice (Ritzmann & Tabakoff, 1976b). We have therefore examined serotoninergic function in brains of mice physically dependent on ethanol by methods which do not involve the use of drugs which in themselves may alter serotoninergic activity. We also attempted to separate the serotoninergic system into areas rich in cell bodies (brainstem) and those containing the neuronal processes of these cell bodies (forebrain) (Dahlström & Fuxe, 1965; Mandel, Knapp & Hsu, 1974).

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MATERIALS AND METHODS

Male C57Bl/6 mice, $\sim$ 25 g, purchased from ARS Sprague Dawley, were kept in our laboratories at least 7 days before being randomly assigned to either the ethanol or control diet groups. All animals were maintained at 22 $\pm$ 1° with a 600–1800 h light cycle throughout the experiments. At the onset of each experiment, all mice were placed in individual cages and offered a liquid diet consisting of Carnation Slender with 100 g litre^{-1} sucrose and 3 g litre^{-1} Vitamin Diet Fortification Mixture (ICN Pharmaceuticals Inc.). On the 2nd day of the experiment, one group of mice was offered the liquid diet in which the sucrose was replaced with 7% v/v ethanol U.S.P., while the second group of mice was continued on the diet containing sucrose. Since animals offered the ethanol-containing diet consumed 10 $\pm$ 3 ml daily, control animals received only 12 ml of the sucrose-containing diet per day. Weight, amount of diet consumed, behavioural intoxication, body temperature (Ritzmann & Tabakoff, 1976b) and blood ethanol concentrations (Tabakoff, Anderson & Ritzmann, 1976) were monitored during the chronic administration of ethanol.

After 7 days of consuming the ethanol-containing diet, mice were again given the sucrose-containing diet (withdrawal). All mice were injected 7–8 h after withdrawal with [3[1]–^{14}C] L-tryptophan (New England Nuclear, 0·1 mCi/0·4 mg). Each mouse received 50 μCi kg^{-1} by intraperitoneal (i.p.) injection. Samples were taken for determination of blood ethanol concentrations (Tabakoff & others, 1976), and body temperature and withdrawal signs* were determined and recorded at 2-h intervals after withdrawal of ethanol. In certain experiments, mice received pargyline HCl (Saber Laboratories), (75 mg kg^{-1}, i.p.) instead of [^{14}C]tryptophan.

After injection with [^{14}C]tryptophan or pargyline, mice were decapitated at intervals noted in Figs 1–4, and the brains rapidly removed and separated into brainstem and forebrain by a coronal section extending from the rostral border of the superior colliculi to the caudal border of the mamillary bodies. The cerebellum was discarded. Mixed arterial and venous blood was collected from the neck in heparinized centrifuge tubes. Blood and brain parts from 4 mice were pooled for analysis. Plasma was separated by centrifugation and an ultrafiltrate prepared as described by Knott & Curzon (1972) by use of Amicon 'Diaflo' filters. Brain tryptophan, 5-HT and 5-HIAA were separated by column chromatography on Dowex 50, Amberlite CG 50 and Sephadex G-10 respectively (Tabakoff, Ritzmann & Boggan, 1975; Tabakoff & Moses, 1976). Prior experiments using radioactive 5-HT, tryptophan and 5-HIAA at concentrations approximating those found in samples showed complete separation of these compounds could be achieved. The only exception was a contamination of the fraction containing 5-HIAA eluting from the Sephadex G-10 columns with 1 $\pm$ 0·5% of the tryptophan counts. All values for radioactive 5-HIAA have, therefore, been adjusted to account for this contamination.

Aliquots of fractions containing tryptophan, 5-HT and 5-HIAA which were eluted from the chromatographic columns, were assayed for these compounds as described below. Separate aliquots of each fraction were lyophylized in counting vials. The residue was redissolved in 0·1 ml of 0·5 N ammonium hydroxide; 15 ml Bray's solution (Bray, 1960) was added, and radioactivity was determined using a Beckman LS 350 scintillation counter. Efficiency of counting was established by use of internal standards and quenching curves. Tryptophan concentrations in brain, as well as in plasma and ultrafiltrates of plasma, were determined using a modification (Bloxam & Warren, 1974) of the method of Denckla & Dewey (1967). 5-HT and 5-HIAA concentrations were determined using a modification of the method of Maickel & Miller (1966). For the assay of 5-HIAA, samples were divided into 2 aliquots: one ('blank') was treated with 0·04% sodium periodate before being assayed; the other was protected by addition of 2% cysteine before the periodate; both aliquots were assayed using 0·1% *o*-phthalaldehyde in absolute ethanol. For the assay of 5-HT, *o*-phthalaldehyde, 0·5% in absolute ethanol, was used. All samples were adjusted for recovery by use of standards simultaneously carried through procedures.

Statistical significance of results was ascertained by use of the Students' *t*-test and regression lines were determined and compared by computer.

RESULTS

We have previously published an extensive description of the behaviour and physiological state of mice chronically consuming a liquid diet containing 7% ethanol (Ritzmann & Tabakoff, 1976b), mice used in the current experiments behaved similarly. They showed behavioural signs of intoxication (i.e. characteristic gait, loss of equilibrium) beginning on the 2nd day of drinking. These signs intensified and extended throughout the phase of alcohol intake. Blood alcohol concentrations measured on the morning of the 6th and 7th days of ethanol consumption were 340 $\pm$ 60 (n = 6) and 285 $\pm$ 56 (n = 6) mg% respectively. Ethanol was undetectable within 4–6 h after its removal from the diet, and at the time of injection of either [^{14}C]tryptophan or pargyline, the mice were exhibiting typical signs of ethanol withdrawal (Ritzmann & Tabakoff, 1976b). The proportion of animals in each category of behavioural symptoms of withdrawal was as follows: 0: 0·03, 1: 0·37, 2: 0·44, 3: 0·16, 4: 0·0 (n = 42). Their mean temperature at the time of the experiments was 33·3 $\pm$ 2·7 (sucrose control mice had a temperature of 37·6 $\pm$ 0·3, n = 12, none exhibited withdrawal signs). We have previously demonstrated that hypothermia is a good measure of the severity of the withdrawal syndrome (Ritzmann & Tabakoff, 1976b).

* Behavioural signs of withdrawal were rated as follows: mice received a score of 0 if there were no overt signs of withdrawal; a score of 1 if they exhibited no symptomatology until lifted by the tail and then exhibited characteristic tremors and spasms; a score of 2 if they exhibited spontaneous spasms; 3 points if they exhibited spontaneous spasms and spontaneous clonic seizures, and 4 points if they had spontaneous clonic and tonic seizures.

Table 1. *Effect of withdrawal from chronic ethanol treatment on brain tryptophan, 5-HT, and 5-HIAA and on plasma tryptophan**.

	Ethanol-treated	Control
Tryptophan		
Forebrain	11·4 ± 0·67 (n = 20)	11·7 ± 0·57 (n = 18)
Brainstem	12·6 ± 0·78 (n = 20)	13·8 ± 0·87 (n = 18)
5-HT		
Forebrain	4·2 ± 0·14 (n = 18)	4·5 ± 0·19 (n = 18)
Brainstem	5·6 ± 0·33 (n = 18)	5·6 ± 0·45 (n = 18)
5-HIAA		
Forebrain	1·8 ± 0·09 (n = 20)	2·0 ± 0·16 (n = 20)
Brainstem	3·7 ± 0·07 (n = 20)	3·6 ± 0·24 (n = 18)
Plasma tryptophan		
Total	112·6 ± 4·07 (n = 16)	99·8 ± 4·43 (n = 16)
Free	13·3 ± 1·53 (n = 16)	18·4 ± 2·20 (n = 16)

* Values expressed as mean ± s.e.m., nmol g^{-1} tissue or nmol ml^{-1} plasma, were obtained from C57B1/6 mice killed between 7 and 8½ h after withdrawal of ethanol (ethanol-treated). Control groups of animals were killed simultaneously. Since no differences in values were evident between mice killed at different intervals within this time, all values were pooled. n represents the number of groups assayed and each group consisted of four mice.

Table 1 summarizes the concentrations in plasma of total and free (ultrafiltrable) tryptophan as well as those of tryptophan, 5-HT and 5-HIAA in forebrain and brainstem. There were no significant differences ($P > 0{\cdot}1$) in the concentrations of 5-HT, tryptophan, or 5-HIAA between mice undergoing withdrawal and control animals. Although tryptophan concentrations were similar in forebrain and brainstem, higher concentrations of 5-HT and 5-HIAA were present in brainstem compared to forebrain in both the ethanol-treated and control mice. No significant differences between treated and control mice were evident in the total or ultrafiltrable tryptophan concentrations in plasma and its concentrations in brain and plasma were similar to those reported by Tabakoff & Moses (1976) in untreated C57B1/6 animals consuming Purina Lab Chow and having free access to water.

No significant differences in the accumulation of 5-HT could be found between ethanol-treated and control mice when they were injected with pargyline 6 h after ethanol withdrawal in the treated animals (Table 2).

Table 2. *Accumulation of 5-HT in brainstem after treatment with pargyline**.

Time after pargyline (min)	5-HT (nmol g^{-1} brain) Control	Ethanol-treated
0	5·5 ± 0·16	5·3 ± 0·37
60	7·8 ± 0·29	8·2 ± 0·45

* Pargyline (75 mg kg^{-1}) was administered intraperitoneally 6 h after ethanol was removed from the diet of C57B1/6 mice drinking such a diet for seven days. Animals drinking the control, sucrose-containing diet were similarly injected with pargyline. Brainstem 5-HT concentrations were determined immediately after (0 time) or 60 min after the injection of pargyline. Six mice were used to derive the mean ± s.e.m.

On the other hand, when mice were injected with [^{14}C]tryptophan, the accumulation of radioactivity in tryptophan, 5-HT and 5-HIAA pools in brains of ethanol-treated mice undergoing withdrawal differed from such accumulation in the control animals (Figs 1, 2 and 3). The accumulation and decline of labelled 5-HT in brain, coupled with the accumulation of radioactive 5-HIAA during the first 45 min after injection of [^{14}C]tryptophan (Figs 2 and 3), indicated that the rate of formation and utilization of 5-HT was decreased in mice undergoing withdrawal. Differences in the rates of uptake and metabolism of

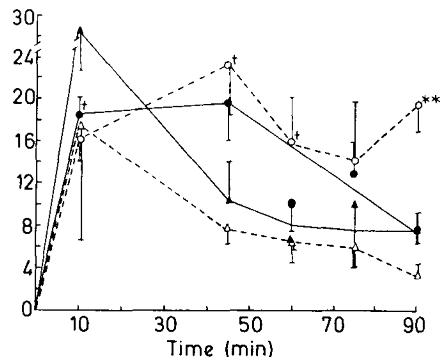

FIG. 1. Accumulation of [^{14}C]tryptophan in brains of ethanol-treated mice undergoing withdrawal and of control mice. Animals were injected with [^{14}C]tryptophan (50 μCi kg^{-1}, i.p.), killed at the times indicated, and forebrain and brainstem were analysed for [^{14}C]-tryptophan. Values are expressed as mean d min^{-1} g^{-1} ± s.e.m. For details of ethanol treatment and tryptophan analysis, see text. The following symbols apply to Figs 1, 2 and 3: Brainstem, ethanol-treated (●–●). Brainstem, control (▲–▲). Forebrain, ethanol-treated (○--○). Forebrain, control (△--△). † = $0{\cdot}05<P<0{\cdot}1$; †† = $0{\cdot}025<P<0{\cdot}05$; ✱ = $0{\cdot}01<P<0{\cdot}025$; ✱✱ = $0{\cdot}005<P<0{\cdot}01$. Ordinate—[^{14}C]Tryptophan (d min^{-1} × 10^{-3} g^{-1} tissue). Abscissa—Time after injection (min).

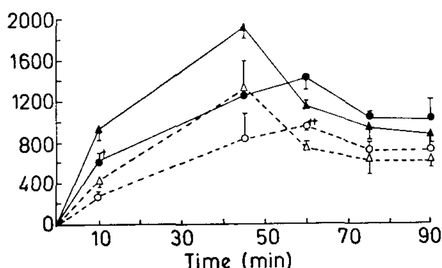

FIG. 2. Accumulation of ^{14}C-5-HT in brains of ethanol-treated mice undergoing withdrawal and control mice. See Legend to Fig. 1 for details. Ordinate—^{14}C-5-HT (d min^{-1} g^{-1} tissue). Abscissa—Time after injection (min).

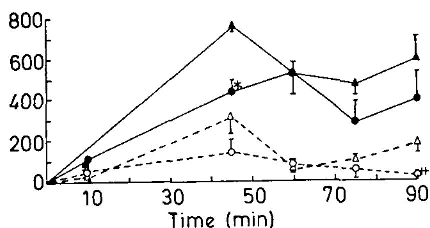

FIG. 3. Accumulation of ^{14}C-5-HIAA in brains of ethanol-treated mice undergoing withdrawal and control mice. For details see Legend to Fig. 1. Ordinate —^{14}C-5-HIAA (d min^{-1} g^{-1} tissue). Abscissa—Time after injection (min).

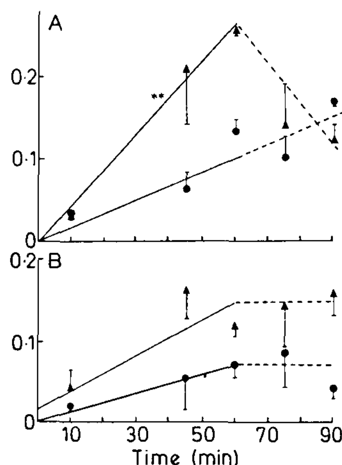

FIG. 4. Time course of accumulation of ^{14}C-5-HT in brainstem (A) and forebrain (B) of ethanol-withdrawn (●–●) and control (▲–▲) mice. To adjust for the availability of [^{14}C]tryptophan in brain values are expressed as ^{14}C-5-HT (d min^{-1} g^{-1})/[^{14}C]tryptophan (d min^{-1} g^{-1}). Solid lines (0–60 min) are regression lines calculated using computer analysis. The slopes of the regression lines calculated for brainstem ^{14}C-5-HT are significantly different ($P<0.001$**) for ethanol-treated and control animals. Ordinate—^{14}C-5-HT/[^{14}C]tryptophan. Abscissa—Time after injection (min).

[^{14}C]tryptophan between control and ethanol-withdrawn mice would contribute to differences in the concentrations of radioactive tryptophan found in brain at the time of assay (Fig. 1). Several authors have indicated that the content of labelled tryptophan in brain should be taken into account when accumulation of radioactive 5-HT is to be considered (Lin, Costa & others, 1969; Schubert, Nybäck & Sedvall, 1970; Shields & Eccleston, 1972). Fig. 4 illustrates the accumulation and decline of radioactive 5-HT adjusted for the availability of its precursor in forebrain and brainstem. When results were expressed as the ratio of ^{14}C-5-HT/[^{14}C]tryptophan in brainstem during the first 60 min after injection of [^{14}C]tryptophan, highly significant (t value = 6·7, d.f. = 14) differences in regression lines fitted to these data were obtained for withdrawn compared with control animals. Although a similar pattern was obtained with data from the forebrain of mice undergoing withdrawal compared with control animals (Fig. 4), the differences in the slope of regression lines were not statistically significant (t = 1·4, d.f. = 14).

DISCUSSION

After chronic ethanol ingestion followed by a period of abstinence, animals (Mello, 1973; Goldstein, 1975) and man (Gross, Lewis & Hastey, 1974) experience a withdrawal syndrome which reaches its most severe stages as ethanol is eliminated from the body and includes convulsions and changes in body temperature (Hunt & Majchrowicz, 1974b; Ritzmann & Tabakoff, 1976b). The neurochemical correlates determining these symptoms are at present obscure, although changes in neuronal function, which are reflected in changes in the metabolism of neurotransmitter substances, probably contribute to the expression of the withdrawal syndrome.

Previous attempts aimed at examining the 'turnover' of 5-HT in brains of animals treated chronically with ethanol have utilized monoamine oxidase inhibitors and monitored the accumulation of 5-HT or the decline of 5-HIAA (Neff & Tozer, 1968). Several pitfalls are inherent in this approach. The commonly used monoamine oxidase inhibitor, pargyline, has been shown to block the firing of serotoninergic neurons (Aghajanian & others, 1970). This effect, which may be mediated by the increased concentrations of 5-HT (Aghajanian, Bunney & Kuhar, 1973) evident after inhibition of monoamine oxidase, would result in changes in utilization of 5-HT and

could obscure changes produced by the chronic ethanol treatment. We have also demonstrated that pargyline lowers body temperature in control and ethanol-withdrawn mice (Ritzmann & Tabakoff, 1976a), and this effect may differentially influence results since temperature-regulating mechanisms are altered by chronic consumption of ethanol. In addition, we have demonstrated (Tabakoff & others, 1975) that ethanol can interfere with the transport of 5-HIAA from brain, a factor which should be considered when 5-HT turnover is to be monitored.

On the other hand, rates of 5-HT synthesis in drug-treated and control animals can be compared by monitoring the accumulation of radioactive amine in brain after administration of labelled tryptophan (Lin & others, 1969; Schubert & others, 1970; Shields & Eccleston, 1972). In such studies, care should be taken not to interfere with endogenous concentrations of amines or their precursors. Comparison of concentrations of free and bound plasma and brain tryptophan obtained in current studies with concentrations in mice not injected with [^{14}C]tryptophan (Tabakoff & Moses, 1976) indicated that the injection of the radioactive amino acid did not alter the concentrations or distribution of tryptophan in plasma or brain.

The estimation of 5-HT 'turnover' (Neff & Tozer, 1968) by the use of pargyline during the withdrawal period indicated no difference in turnover between treated and control animals (Table 2) (Frankel & others, 1974; Hunt & Majchrowicz, 1974a). However, estimation of serotoninergic function, using the initial rates of accumulation of radioactive 5-HT and 5-HIAA derived from [^{14}C]tryptophan indicated that mice undergoing withdrawal have a significantly lower rate of 5-HT utilization in brainstem as well as different patterns of [^{14}C]tryptophan accumulation and catabolism (Figs 1–4).

The decreased rate of 5-HT metabolism in the brainstem was indicated by the lower rate of accumulation of radioactive 5-HT and 5-HIAA during the early period (0–45 min) after the injection of [^{14}C]-tryptophan. Since the accumulation of [^{14}C]tryptophan in brains of animals undergoing withdrawal differed from that of control animals, we adjusted the amount of ^{14}C-5-HT that accumulated in brain to account for differences in availability of precursor for ^{14}C-5-HT synthesis by expressing the results as the ratio of ^{14}C-5-HT/[^{14}C]tryptophan (Shields & Eccleston, 1972) (Fig. 4). Differences in the accumulation of [^{14}C]tryptophan in brains of ethanol-treated and control mice may have been due to different rates of its absorption from the peritoneum of the animals, differences in its availability for uptake into brain or altered mechanisms of active transport due to chronic ethanol exposure.

Rates of accumulation of ^{14}C-5-HT appeared lower in forebrain in ethanol-withdrawn animals compared with control mice but were not statistically significant (Fig. 4). That highly significant differences were obtained in brainstem but not forebrain may be a result of both the different rates of accumulation of [^{14}C]tryptophan (Fig. 1) and the different capacities of the two areas to synthesize 5-HT (Glowinski, Hamon & Héry, 1973; Meek & Neckers, 1975). In addition, since electrophysiological measurements indicate that the reticular formation is more profoundly affected by acute administration of ethanol compared with most forebrain structures (with the exception of the associative cortex) (Mendelson, 1970; Kalant, 1975), the chronic administration of ethanol may also result in greater changes in neural activity in the brainstem region.

5-HT turnover in brain has been shown to be sensitive to both the concentrations of precursor (tryptophan) (Fernstrom & Wurtman, 1971) and to the electrical activity of the serotoninergic neurons (Sheard & Aghajanian, 1968). Although the uptake of [^{14}C]tryptophan into brain was altered in ethanol-withdrawn mice (Fig. 1), such differences did not account for differences in initial accumulation of ^{14}C-5-HT in brains of ethanol-withdrawn and control mice (Fig. 4).

We would therefore postulate that decreases in utilization of 5-HT witnessed in mice withdrawn from ethanol are probably due to changes in the electrical activity of the serotoninergic neurons during withdrawal. Decreased activity of these neurons has been shown to predispose animals to drug- and sound-induced seizures (Schlesinger & others, 1968), and consonant with such observations, results from our laboratory (Tabakoff & Boggan, 1974) and those of others (Freund & Walker, 1971) have demonstrated that both mice and rats are significantly more susceptible to such seizures during withdrawal. However, the possibility that decreased serotoninergic activity is the factor responsible for certain symptoms of withdrawal requires further exploration.

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