Pharmacology Biochemistry & Behavior, Vol. 10, pp. 113–119. Printed in the U.S.A.

Physical Dependence Following Prolonged Ethanol or *t*-Butanol Administration to Rats[1]

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(Received 7 August 1978)

WOOD, J. M. AND R. LAVERTY. *Physical dependence following prolonged ethanol or t-butanol administration to rats.* PHARMAC. BIOCHEM. BEHAV. 10(1) 113–119, 1979.—Rats were fed on liquid diets containing ethanol or *t*-butanol. On removal of either alcohol from the diet after 4–20 days, withdrawal reactions were observed. The range of withdrawal signs produced by the two alcohols were identical although the time course of the withdrawal reactions differed. Administration of one alcohol prevented the appearance of a withdrawal reaction in rats dependent on the other alcohol. Depletion of brain noradrenaline and dopamine did not prevent the development of physical dependence on either alcohol. Thus, neither aldehyde formation nor brain catecholamines appear to be involved in the development of physical dependence on these alcohols.

Ethanol *t*-Butanol Alcohol Physical dependence Withdrawal Catecholamines 6-Hydroxydopamine

IT HAS BEEN well established that physical dependence on ethanol may develop after periods of prolonged ethanol intake in man and many animal species [11]. However, as yet, little is known about the mechanisms involved in the development of ethanol dependence.

It has been proposed that ethanol dependence is the result of the formation of psychoactive compounds from acetaldehyde, the main metabolite of ethanol, and monoamines in the central nervous system (CNS) [3, 5, 6]. Competition between acetaldehyde and the aldehyde metabolites of the monoamines for the enzyme aldehyde dehydrogenase may result in the condensation of the aldehydes with the monoamines to form dependence-producing substances. The formation of these proposed compounds from ethanol and monoamines has been demonstrated in vitro [2, 5, 6] and in vivo after ethanol administration when acetaldehyde [4] or dopamine [4,24] concentrations were artificially elevated. However, these compounds have not been detected when ethanol is administered under normal conditions.

Tetrahydropapaveroline (formed from 3,4-dihydroxyphenylacetaldehyde and dopamine) and salsolinol (formed from acetaldehyde and dopamine) are reported to increase preference for ethanol when chronically infused into the cerebral ventricle of the rat [18,20]. These observations appear to support the hypothesis implicating a role for these compounds in ethanol dependence. However, tetrahydropapaveroline and salsolinol have been shown to be formed in humans during levodopa administration with and without concomitant ethanol consumption [23], and if these compounds were involved in ethanol dependence, it might be expected that administration of levodopa would intensify ethanol dependence or itself produce dependence. Yet, neither of these possibilities has been reported to occur.

Support for hypotheses implicating acetaldehyde in ethanol dependence comes from the observation that after prolonged exposure to either acetaldehyde or ethanol vapour, mice exhibit similar behavioural changes on withdrawal [21]. However theories implicating acetaldehyde are incompatible with the observation that withdrawal reactions after chronic administration of either ethanol or *t*-butanol to rats appear identical [26]. *t*-Butanol (2-methyl-propan-2-ol) is a tertiary alcohol which unlike ethanol, cannot be metabolised to an aldehyde [12,27]. Also, in experiments where ethanol is administered by inhalation to mice, co-administration of pyrazole, an alcohol dehydrogenase inhibitor, does not decrease the intensity of withdrawal seizures although it inhibits acetaldehyde formation [10].

In order to confirm the observations of Wallgren, Kosunen and Ahtee [26] we have compared the physical dependence produced by prolonged ethanol administration with that produced by prolonged *t*-butanol administration. Preliminary results have been reported previously [29]. The present report extends our previous observations and also investigates the possible involvement of brain catecholamines in the development of alcohol dependence.

METHOD

Animals

Random-bred male albino rats of Wistar origin were used. Rats initially weighed 120 ± 5 g and were aged 6–7 weeks. They were housed individually or in pairs.

[1]This work was supported by the Medical Research Council of New Zealand. Excellent technical assistance was provided by Mrs. Jan Cowan.

Induction of Physical Dependence on Ethanol

Chronic ingestion of ethanol was achieved by maintaining rats on a completely liquid diet as their sole source of calories and fluid [9]. The liquid diet consisted of Complan (Glaxo Laboratories [N.Z.] Ltd.) (188 g/l) and ethanol (95% v/v, 87ml/l). Twelve rats were allowed continuous access to the ethanol-containing liquid diet which was offered in graduated drinking cylinders. The amount of diet consumed was recorded daily and fresh diet administered.

Six control rats were weight-matched as closely as possible with the ethanol-fed rats. Control diets were the same as the ethanol diets except that sucrose was isocalorically substituted for ethanol. Control rats were given the same quantity of diet as the ethanol-fed rats had consumed on the previous day.

The diurnal feeding pattern of rats with continuous access to either the ethanol or sucrose diets was recorded by measuring the pressure drop at the tip of the feeding cylinder as fluid was consumed from the cylinder.

After 20 days, rats on the ethanol diet were withdrawn from ethanol by replacing the ethanol diet with the sucrose diet. Rats were carefully observed for up to 9 hours for signs of a withdrawal reaction. The response to audiogenic stimuli was assessed by rattling keys over the top of the cages. Rats were observed at 24, 48 and 72 hours after withdrawal for persisting signs of withdrawal.

Induction of Physical Dependence on t-Butanol

Twelve rats were fed as described above on a liquid diet consisting of Complan (188 g/l), sucrose (111 g/l) and *t*-butanol (20 ml/l). The amount of *t*-butanol added was decided by determining the concentration of *t*-butanol which resulted in a similar food intake to that of the ethanol-containing diet.

After 20 days the rats were withdrawn from *t*-butanol by removal of the *t*-butanol from the diet and observed for signs of a withdrawal reaction as described above.

Estimation of Blood Ethanol and t-Butanol Concentrations

Blood samples were taken at the same time of day (1600 hours) for blood alcohol estimations 1, 5 and 15 days after starting the alcohol-containing diets. On Day 10 samples were taken at 0930, 1300, 1600 and 2000 hours. Samples were also taken at approximately hourly intervals after withdrawal.

Blood samples were collected into 50 μl heparinized capillary tubes from small incisions in the lateral tail vein of unanaesthetised, restrained rats, warmed at 30°C for 10 min. Blood ethanol and *t*-butanol concentrations were determined on diluted blood samples (1:20) by gas chromatography. A Pye series 104 gas chromatograph with a flame ionization detector was used. The column (glass, 1 m×4 mm, i.d.) was packed with Chromosorb 102. The column temperature was 160°C and the carrier gas (N_2) flow rate was 35 ml/min. *t*-Butanol was used as the internal standard for the ethanol assay and vice versa.

Time Course of the Onset of Physical Dependence

Twenty-four rats were fed the ethanol-containing diet and 24 fed the *t*-butanol-containing diet. Four animals in each group were withdrawn after 4, 6, 8, 10, 12 and 14 days on the diets and closely observed for signs of a withdrawal reaction.

Cross-dependence Between Ethanol and t-Butanol

Cross-dependence could not be tested simply by interchanging the diets of ethanol- and *t*-butanol-dependent rats as it was found that the dependent animals would not drink the different diet initially. Therefore it was necessary to administer the appropriate alcohol by IP injection after withdrawal of the diet.

Ten rats were maintained on the ethanol-containing diet and 10 on the *t*-butanol-containing diet for 14 days. Six rats were given 3 IP injections of *t*-butanol (7 mmol/kg, 10% v/v in 9 g/l NaCl [saline]); the first injection was given immediately after withdrawal from ethanol and the others at 3 and 6 hours later. Similarly, 6 rats were given ethanol (43 mmol/kg, 20% v/v in saline) after withdrawal from *t*-butanol.

The remaining 4 rats from each group were injected with equivalent volumes of saline after withdrawal. All rats were observed for signs of withdrawal up to 8 hours after withdrawal.

Effects of 6-Hydroxydopamine on Physical Dependence

Sixteen rats were treated with 6-hydroxydopamine (6HD). 6HD (0.8 μmol in 20 μl of saline) was injected into the left lateral ventricle of lightly anaesthetised rats using a stereotaxic technique [15]. As controls, 10 rats were injected intraventricularly with 20 μl of saline. One week after the treatment, 10 of the 6HD-treated rats were started on the ethanol-containing diet. The 10 saline-treated rats were pair-fed with the 6HD-treated rats on the ethanol-containing liquid diet. The other six 6HD-treated rats were fed on the *t*-butanol containing liquid diet.

After 2 weeks the rats were withdrawn from ethanol or *t*-butanol and observed for signs of a withdrawal reaction.

One week after withdrawal all rats were killed and their brains removed for brain catecholamine estimations. Brain tissue concentrations of noradrenaline and dopamine were estimated fluorimetrically by the method of Laverty and Taylor [14].

RESULTS

Induction of Physical Dependence on Ethanol

Over 20 days, the mean daily ethanol intake of individual rats ranged from 258–373 mmol/kg (mean = 320, n = 12). Daily ethanol intake of individual rats was variable. Periods of spontaneous reduction in diet intake were observed with a few rats.

The addition of ethanol to the diet resulted in a more even food consumption over a 24 hour period. Rats with continuous access to the ethanol-containing liquid diet consumed approximately 55% of the diet overnight, whereas rats allowed continuous access to the sucrose-containing liquid diet consumed approximately 65% of the diet overnight (2030–0830 hr).

The rats maintained on the ethanol-containing diet were not obviously intoxicated. However all animals were docile and very easy to handle. Slight ataxia was observed in a few rats. The mean and range of blood ethanol concentrations measured while the rats had access to the ethanol diet are given in Table 1.

Two to 4 hours after removal of the ethanol-containing diet, all withdrawn rats had begun to develop signs of a withdrawal reaction. Initial signs included excessive exploration of the cage, head-bobbing and forepaw grooming. The ani-

TABLE 1

BLOOD ALCOHOL CONCENTRATIONS (MEAN AND RANGE) IN RATS FED ETHANOL- OR *t*-BUTANOL-CONTAINING DIET

Alcohol	Blood Alcohol Concentration (mmol/l) at 1600 hr on days			during day 10 at			
	1	5	15	0930 hr	1300 hr	1600 hr	2000 hr
Ethanol	26	49	53	42	39	25	40
n=12	(18–55)	(30–63)	(16–74)	(29–59)	(15–61)	(17–38)	(27–70)
t-butanol	12	11	17	20	19	16	16
n=11	(7–18)	(7–18)	(6–24)	(15–25)	(11–23)	(6–23)	(2–20)

mals became progressively more irritable and hyperreactive and developed piloerection, muscular rigidity, stiff-curled tails, a broad-based gait, and tremor. As the withdrawal reaction developed further the rats became hypoactive and assumed abnormal rigid postures. Three rats which had spontaneously reduced their ethanol intake a few days prior to withdrawal had only very mild signs of withdrawal. In the other 9 rats signs were more severe. Rats with the more severe withdrawal reactions vocalised when handled and some vocalised spontaneously.

Spontaneous convulsions were observed in 3 rats. The convulsive activity was clonic and involved only the forepaws while the animal was reared on its hind-limbs. This convulsive activity was accompanied by excessive salivation and followed by 'wet-dog' shaking. All ethanol-withdrawn rats were more disturbed by the audiogenic stimulus than the sucrose-fed control rats. A few of the ethanol-withdrawn rats were induced to run frantically around their cage. Four of these rats then exhibited whole-body, tonic-clonic convulsions. The initial convulsion was usually followed by a series of hopping movements and vocalisations. In some animals further whole body convulsions followed the hopping phase.

Some withdrawal signs (irritability and muscular rigidity) persisted for up to 48 hr after withdrawal. Each rat was given a withdrawal severity rating depending on the type and severity of the withdrawal signs observed (Table 2). Ratings ranged from 0–6 in order of increasing withdrawal severity (Table 2).

Audiogenic convulsions could not be induced in any of the sucrose-fed control rats. None of the behavioural changes observed in the ethanol-withdrawn rats were apparent in the control rats.

Blood ethanol concentrations measured immediately after removal of the ethanol diets ranged from 14 to 62 mmol/l. Concentrations fell rapidly and were below the limit of detection (2 mmol/l) in all rats after about 5 hr. Withdrawal signs were apparent while ethanol was still detectable in the blood. The initial period of hyperactivity occurred while

TABLE 2

WITHDRAWAL SEVERITY RATINGS AFTER 4–20 DAYS ON SUCROSE-, ETHANOL- OR *t*-BUTANOL-CONTAINING DIET

Diet	Days on Diet	Number of Rats Given Withdrawal Severity Rating* of						
		0	I	II	III	IV	V	VI
Sucrose	20	6	0	0	0	0	0	0
Ethanol	4	0	1	0	0	2	1	0
	6	0	2	0	2	0	0	0
	8	0	0	1	2	1	0	0
	10	0	0	1	2	0	0	1
	12	0	0	0	2	0	0	2
	14	0	0	0	1	0	0	3
	20	0	3	2	3	0	2	2
t-Butanol	4	0	0	2	0	0	2	0
	6	0	0	1	1	1	1	0
	8	0	0	0	2	0	0	2
	10	0	0	0	2	0	1	1
	12	0	0	0	3	0	0	1
	14	0	0	0	2	0	1	1
	20	0	2	0	1	1	3	4

*Rating: (0) No withdrawal signs apparent, (1) Mild signs of muscular rigidity and irritability, (II) Moderate signs of muscular rigidity and irritability, tail signs, abnormal gait, (III) Pronounced signs of muscular rigidity and irritability, curled tail, tremors, twitching or shaking, (IV) Frantic running in response to audiogenic stimuli, (V) Single spontaneous or audiogenic convulsions, (VI) Multiple convulsive seizures.

blood ethanol concentrations were falling and the animals became hypoactive when ethanol was no longer detectable in the blood. The spontaneous forelimb convulsions were observed just before blood ethanol concentrations fell below detectable levels. Audiogenic convulsions were induced when ethanol was no longer detectable. Blood ethanol clearance and the time of onset of various withdrawal signs of a representative rat are shown in Fig. 1.

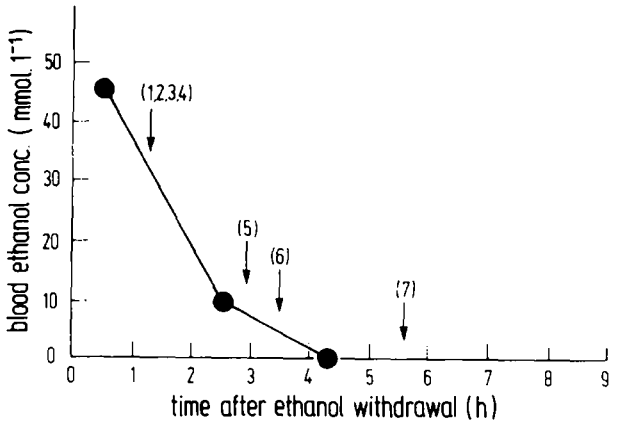

FIG. 1. Blood ethanol elimination and time of onset of various withdrawal signs for a representative rat undergoing ethanol withdrawal. Signs: (1) head-bobbing, excessive grooming, and paw-shaking, (2) irritability, (3) abnormal gait, (4) tail signs, (5) spontaneous forelimb convulsion, (6) hypoactivity, (7) audiogenic convulsion.

Induction of Physical Dependence on t-Butanol

Over 20 days, the mean daily *t*-butanol intake of individual rats ranged from 44–54 mmol/kg (mean=47, n=11). One rat was withdrawn from the experiment because it refused to eat. Daily *t*-butanol consumption was variable and periods of spontaneous abstinence were observed in a few rats. As with the ethanol liquid diet, approximately 55% of the *t*-butanol diet was consumed overnight.

Signs of intoxication observed in the rats while on the *t*-butanol-containing diet included docility and slight ataxia.

Mean and range of blood *t*-butanol concentrations while the rats had access to the *t*-butanol diet are shown in Table 1. One rat withdrew spontaneously from *t*-butanol on Day 10. This is reflected in the increased range of blood *t*-butanol concentration with time. Signs of irritability, hyperreactivity and muscular rigidity were observed in this rat.

Removal of *t*-butanol from the diet resulted in the appearance of a variety of withdrawal signs. The range of withdrawal signs observed was the same as that described for ethanol withdrawal but the time course and severity of the signs differed. Animals initially appeared intoxicated. Head-bobbing and paw-shaking were first observed in some rats 3–4 hr after withdrawal. Muscular rigidity, tail signs, abnormal gait, tremor and irritability became apparent after 5–6 hr.

Two rats which had spontaneously reduced their *t*-butanol intake 1 to 2 days prior to withdrawal showed only mild signs of withdrawal (irritability and muscular rigidity). In the remaining 9 animals, withdrawal signs were more severe. Muscular rigidity, tremor and tail signs were pronounced and the rats became irritable and hyperreactive. All these rats vocalised when handled. Four rats had spontaneous forelimb convulsions. Five had audiogenic convulsions and 3 of these rats died as a consquence.

In the surviving rats signs of irritability, hyperreactivity and muscular rigidity were considerable at 24 hr. These signs persisted in some animals for up to 72 hr, but became progressively less obvious each day. Withdrawal severity ratings (0–6) were assigned to each *t*-butanol-withdrawn rat (Table 2).

The clearance of *t*-butanol from the blood was much slower than the clearance of ethanol. *t*-Butanol was still detectable in all rats 8–9 hr after withdrawal. Blood *t*-butanol clearance and the time of onset of various withdrawal signs of a representative rat are shown in Fig. 2.

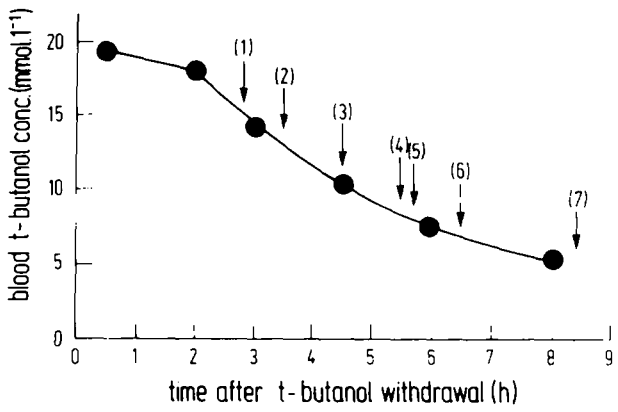

FIG. 2. Blood *t*-butanol elimination and time of onset of various withdrawal signs for a representative rat undergoing *t*-butanol withdrawal. Signs: (1) head-bobbing, excessive grooming and paw-shaking, (2) irritability, (3) abnormal gait, (4) tail signs, (5) spontaneous forelimb convulsions, (6) hypoactivity, (7) audiogenic convulsion.

Time Course of Onset of Dependence

Signs of a withdrawal reaction were observed in rats deprived of either ethanol or *t*-butanol after as little as 4 days of alcohol intake. Withdrawal severity ratings after 4–20 days of ethanol or *t*-butanol intake are given in Table 2. At each of the times shown, ratings for both the ethanol- and *t*-butanol-withdrawn rats were significantly higher than the ratings for the sucrose-fed control rats (Mann-Whitney U test, $p<0.05$). Ratings for the *t*-butanol-withdrawn rats were significantly higher than ratings for the ethanol-withdrawn rats (Mann-Whitney U test, $p<0.05$).

Cross Dependence Between Ethanol and t-Butanol

On withdrawal from ethanol, the 4 rats that had not received doses of *t*-butanol developed signs of a withdrawal reaction. Over the 8 hr observation period, no withdrawal signs were observed in any of the 6 rats that received *t*-butanol apart from slight irritability when given each dose of ethanol.

Similarly, withdrawal signs were observed in the rats withdrawn from *t*-butanol that were not given ethanol. No withdrawal signs were observed in the rats that had received ethanol.

TABLE 3

WITHDRAWAL SEVERITY RATINGS FOR 6HD- OR SALINE-INJECTED RATS WITHDRAWN FROM ETHANOL OR *t*-BUTANOL AFTER 2 WEEKS ON THE ALCOHOL-CONTAINING DIETS

Group	Number of Rats Given Withdrawal Severity Rating* of						
	0	I	II	III	IV	V	VI
Saline-treated, ethanol-withdrawn (n=10)	0	0	3	2	2	1	2
6HD-treated, ethanol-withdrawn (n=10)	0	0	2	2	1	1	4
6HD-treated, *t*-butanol-withdrawn (n=6)	0	0	2	2	0	0	2

*See Table 2.

Effect of 6-Hydroxydopamine on Physical Dependence

On withdrawal from ethanol or *t*-butanol, withdrawal reactions identical to those described in the results of the first experiments were observed in both the 6HD- and saline-treated rats. Withdrawal severity ratings for these rats are given in Table 3. There was no significant difference in the withdrawal severity ratings between the 6HD- and saline-treated animals (Mann-Whitney U test).

The mean whole brain noradrenaline concentration of the 6HD-treated rats (0.63 ± 0.06 nmol/g, n=16) was reduced to approximately 26% of that of the saline-treated controls (2.46 ± 0.08 nmol/g, n=10). The reduction was statistically significant (Student's *t*-test, $p<0.001$). The mean whole brain dopamine concentration of the 6HD-treated rats (0.80 ± 0.06 nmol/g) was reduced to approximately 36% of that of the saline-treated controls (2.22 ± 0.15 nmol/g). This reduction was also statistically significant ($p<0.001$).

DISCUSSION

Physical dependence on ethanol or *t*-butanol was induced in rats by feeding them on a liquid diet containing the alcohol. The dosage of ethanol consumed was similar to those reported by Branchey, Rauscher and Kissin [1] and Hunter *et al.* [13] who also used similar liquid diets. Branchey *et al.* [1] and Hunter *et al.* [13] reduced the body weight of their rats by 25% prior to administration of the liquid diets. The weight reduction is reported to decrease the rate of ethanol metabolism and therefore, as a consequence, facilitate the establishment of ethanol dependence [9]. In the present study, body weights were not reduced before starting the ethanol diets, yet ethanol intake and the severity of the withdrawal reactions were similar to those reported by the above authors. Thus, it appears that the weight reduction is not necessary and may introduce nutritional complications into an experiment.

The periods of spontaneous abstinence observed in a few rats on either the ethanol- or *t*-butanol-containing diets, were similar to those reported by Hunter *et al.* [13]. This phenomenon has also been reported to occur in monkeys [7,28] and man [19].

Recordings of diurnal patterns of diet intake showed that the addition of either ethanol or *t*-butanol to the basic liquid diet altered the normal diurnal feeding pattern of the rats. When either alcohol was added to the diet, intake was spread more evenly over a 24 hr period. This is probably because the intoxicating properties of the alcohol-containing diet reduced the volume that could be consumed at any one time and therefore more drinking episodes occurred. This effect probably contributes significantly to the success of this method of inducing physical dependence, since it appears that continuously, rather than periodically, elevated blood ethanol concentrations are most effective in producing physical dependence [8, 11, 13, 16]. Measurement of blood alcohol concentrations confirmed that concentrations were continuously elevated in most rats while on the alcohol-containing diet.

Removal of ethanol from the diet resulted in the appearance of a variety of withdrawal signs similar to those reported by other investigators [13, 16, 26]. The range of withdrawal signs observed after withdrawal from *t*-butanol were identical to those seen after ethanol withdrawal and were similar to those described by Wallgren *et al.* [26], after prolonged ethanol or *t*-butanol administration by gastric intubation. The time course of the ethanol and *t*-butanol withdrawal reactions differed, probably because of the difference in the clearance rates of the two alcohols. After ethanol withdrawal blood ethanol concentrations fell rapidly; some withdrawal signs became apparent while blood ethanol concentrations were falling, others when ethanol was no longer detectable in the blood. After *t*-butanol withdrawal blood *t*-butanol concentrations fell more slowly, the time of onset of the various withdrawal signs was longer and the appearance of the signs was more spaced out than after ethanol withdrawal.

Withdrawal reactions produced by *t*-butanol were generally more severe than those produced by ethanol. Physical dependence may develop more readily with *t*-butanol than with ethanol because the much slower rate of *t*-butanol elimination facilitates the maintenance of elevated blood alcohol concentrations.

Withdrawal reactions were produced in all rats after as little as 4 days on either the ethanol- or *t*-butanol-containing diet. The rapid onset of physical dependence on ethanol is consistent with other reports where high doses of ethanol

have been continuously administered to rats [16] or mice [10,17].

t-Butanol completely prevented the development of a withdrawal reaction in ethanol-dependent rats. Similarly, ethanol prevented the development of a withdrawal reaction in *t*-butanol-dependent rats. Thus, cross-dependence between ethanol and *t*-butanol appears to be complete. The similarity of the withdrawal reactions and the existence of cross-dependence suggests that the two alcohols produce physical dependence by a common mechanism. Since *t*-butanol is not oxidised, like ethanol, to an aldehyde, it is unlikely that aldehyde formation is involved in the development of physical dependence on ethanol. Thus, it would appear that it is the alcohol per se rather than one of its metabolites that is responsible for inducing physical dependence.

Calculated on a molar basis, *t*-butanol is approximately 7 times more potent than ethanol in its ability to produce physical dependence. *t*-Butanol, when administered acutely, is approximately 4.8 times as potent as ethanol in producing anaesthesia [25]. This similarity in relative potency of the two alcohols in producing anaesthesia and physical dependence suggests that these effects involve a common site of action. The additional potency of *t*-butanol compared with ethanol in producing physical dependence is probably related to its more prolonged effect due to its slower clearance from the body.

The range of withdrawal signs and the severity and time course of the withdrawal reactions observed in 6HD-treated rats after withdrawal from ethanol or *t*-butanol were similar to those observed in untreated rats. Therefore, catecholamine depletion did not prevent the development of physical dependence on either ethanol or *t*-butanol or the expression of the withdrawal reactions. These results are consistent with the observations of Ritzmann and Tabakoff [22]. Depletion of brain noradrenaline by intraventricular injection of adult mice with 6HD did not prevent the development of physical dependence on ethanol. Thus, intact central stores of noradrenaline and dopamine do not appear to be required for the development of physical dependence on alcohols or for the expression of the alcohol withdrawal reaction.

These results suggest that neither acetaldehyde nor brain catecholamines are involved in the development of physical dependence on ethanol. This makes it unlikely that ethanol dependence is the result of the formation of psychoactive compounds from the interaction of ethanol-derived acetaldehyde and brain monoamines.

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