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EFFECTS OF ACUTE AND CHRONIC ETHANOL ADMINISTRATION ON REGIONAL THIAMIN PYROPHOSPHOKINASE ACTIVITY OF THE RAT BRAIN

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Abstract—Thiamin pyrophosphokinase (TPKase) activity was determined in supernatants of cerebral cortex, cerebellum, pons, medulla, hypothalamus and corpus callosum homogenates obtained from normal rats and from rats given ethanol acutely (a single dose of 4.7 g.kg^{-1} body wt) or chronically (4.7 g.kg^{-1} body wt daily for 35 days) by gastric gavage. Regional cell densities (derived from DNA content) and protein contents were also determined. TPKase was detected in all brain regions investigated, the highest activity being found in the cerebellum or in the pons depending on whether it was expressed per mg of protein or per number of cells, respectively. In samples taken following acute ethanol administration protein content was unaffected, while TPKase activity was significantly reduced in the cerebellum, cerebral cortex and hypothalamus at 90 min and in the cerebellum and cerebral cortex at 300 min.

Chronic ethanol intake was associated with a significant decrease in regional cell densities, protein contents and TPKase activity. The addition of ethanol to the incubation medium of normal tissue supernatant caused a dose-dependent inhibition of TPKase activity.

These results suggest that ethanol markedly impairs thiamin cellular utilization, which may result in depression of brain metabolism.

Thiamin pyrophosphokinase (ATP: thiamin pyrophosphotransferase: EC 2.7.6.2) (TPKase) catalyses thiamin phosphorylation to TPP, the biologically active form of thiamin, and plays an important role in regulating the accumulation of the vitamin in tissues.

TPKase activity has been determined in different organs and tissues including liver [1, 2], heart [3], blood cells, [1, 4], enterocytes [5] and whole brain [6, 7]. No information, however, is available on its brain regional distribution or its sensitivity to ethanol. Impaired conversion of thiamin to its active coenzyme thiamin pyrophosphate (TPP) in the liver has been suggested as a possible mechanism of thiamin deficiency in chronic alcoholism [8], but this hypothesis has been criticized recently on the basis of indirect evidence [9].

Here we report the results of a study on the quantitative distribution of TPKase activity in six different regions of rat brain (cerebral cortex, cerebellum, pons, medulla oblongata, hypothalamus and corpus callosum) and its modification by acute and chronic ethanol administration. The effect of ethanol added *in vitro* was also examined. This work is part of a comprehensive study on the dynamics of thiamin and its phosphoesters in the nervous tissue in different conditions [10–12].

MATERIALS AND METHODS

Animals. The animals used were male Wistar rats (350–410 g of body weight) reared on a standard diet [13]. They were sacrificed by decapitation after an overnight starvation with water *ad lib.* Controls were untreated, normal rats reared on the same diet.

Ethanol treatment. Chronic alcoholism was induced by daily administration of 50% ethanol in water (4.7 g ethanol.kg^{-1} body wt.) by gastric gavage for a period of 35 days. Ethanol was given in the morning, and the rats received the standard diet during the whole period of treatment. All animals were sacrificed 24 hr after the last administration of ethanol. In the acute experiments, the same amount of ethanol was given by gastric gavage to two groups of 16 rats each after a 12 hr starvation. The rats of one group were sacrificed after 90 min and those of the other group after 300 min.

Anatomical dissection. The brain regions (cerebral cortex, cerebellum, pons, medulla, hypothalamus and corpus callosum) were dissected as previously described [10].

Determination of TPKase activity. The dissected tissue was homogenized with a Potter–Elvehjem tissue homogenizer in 0.25 M sucrose solution (5 ml.g^{-1}). The tissue was then ultrasonicated for 10 min at 50 kHz and centrifuged at 105,000 *g* for 1 hr and the precipitate was discarded. All procedures were carried out at 0–4°. The TPKase activity was determined in the supernatant by using labeled thiamin according to the procedure described by Sanioto *et al.* [7], with minor modifications. After incubation and heat deproteinization, samples of the incubation medium were centrifuged and the content of synthesized labeled TPP in the supernatant was determined radiometrically after electrophoretical separation according to Patrini and Rindi [14]. In the experiments on the *in vitro* effect of alcohol on

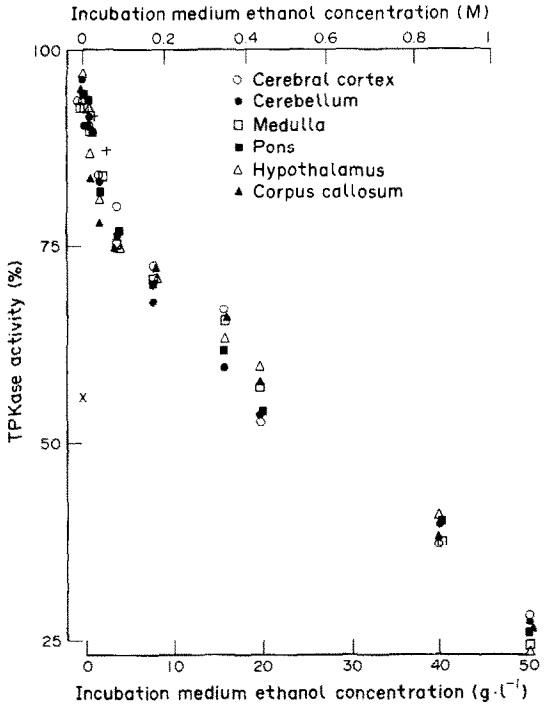

Fig. 1. Effect of ethanol, added to the incubation medium *in vitro*, on TPKase activity in supernatants from different brain regions of the rat. Symbols represent means of 4 experiments, each on triplicate samples. × and +, average of regional TPKase activities after chronic and acute ethanol administration, respectively.

TPKase activity, ethanol was added to the incubation medium in appropriate amounts (Fig. 1).

Cellular density. The total number of cells in the brain regions was estimated by determining tissue DNA content, an approach previously used by several authors to determine cell density in the brain from different species [15–17]. The DNA was extracted according to the procedure described by Zamenhof *et al.* [15] and determined spectrophotometrically [18]. The cell densities were derived from tissue DNA content by assuming that each cell nucleus contains 6.10^{-6} μg of DNA [15, 19]. In preliminary experiments, the recovery of added calf thymus DNA was 102.6% ± 1.73 (mean of 6 experiments ± SE).

Biochemical methods. Proteins were determined according to Lowry *et al.* [20]. Blood ethanol levels were measured by head-space gas chromatography using a column packed with 5% Carbowax 20 M Carbopack B. Nitrogen was used as the carrier gas at a flow-rate of 30 ml min^{-1}. The column was operated isothermically at 90°, while the temperature of the injection and detector ports was 140°. Isopropanol was used as internal standard.

Statistics. The following statistical methods were used for evaluating the significance of the differences of the means for different experimental groups: analysis of variance [21] for the differences among and within groups under all experimental conditions; Dunnett's *t*-test [22, 23], for the comparison between control rats and different groups of treated rats; Newman–Keul's *Q* test, for the comparison between different means in the same experimental condition [21], Student's *t*-test, for the comparison between two single means [24].

Materials. All reagents were of analytical grade and obtained from the sources previously specified [14]. ATP and calf thymus DNA were purchased from Sigma (St. Louis, MO). Thiazole-[2-^{14}C]-thiamin (specific activity, 24.5 mCi.$mmol^{-1}$) was prepared by the Radiochemical Centre (Amersham, U.K.).

The diet was supplied by Piccioni and Co. (Brescia, Italy).

Instruments. The instruments used have been described in a previous paper [14]. In addition, a DU-5 Beckman spectrophotometer (Irvine, CA) and a Felisari (Giussano, Italy) ultrasonic bath were used. The gas chromatograph was a Carlo Erba Fractovap 4200 C (Milan, Italy), equipped with an automatic sampler HS 250 and a flame ionization detector and connected to a central processor (model SP 4000), data interface (model SP 4020) and printer plotter (model SP 4050) Spectra Physics (Mountain View, CA).

RESULTS

Control rats

Cell density. Cellular densities values (Table 1) were generally in good agreement with previous results obtained for the rat brain by determining the DNA level [16] or by direct counting [25]. The cellular density was significantly higher in the cerebellum than in the other regions. The DNA content in whole brain (647.7 ± 7.57 μg.g^{-1} wt: mean ± SE of 3 experiments) was similar to that (636 μg.g^{-1}) reported by Zamenhof *et al.* [15] for the rat.

Protein content. Protein content expressed per g of wet tissue was significantly higher in the hypothalamus and in the corpus callosum than in other brain areas (Table 1). Protein content per cell, expressed as ratio, could be evaluated with some approximation. Cells from different regions appeared to have a similar content of protein except the cerebellum, where protein content was significantly lower (data not shown).

TPKase activity. When expressed per mg of protein, TPKase activity was significantly higher in the cerebellum and pons than in the remaining regions (Table 2). The lowest activity was found in the corpus callosum (74% of the value found in the cerebellum). The value found for the whole brain was 0.63 ± 0.06 nmol TPP.mg^{-1} of protein.hr^{-1} (mean of 7 experiments ± SE), in agreement with that (0.56 ± 0.05; N = 5) reported by Sanioto *et al.* [7]. When cell density was taken into account, the highest mean TPKase activity was found in the pontine cells (Table 2); no statistically significant differences were found among the other areas. The lowest activity was found in cerebellar cells (72% of the value found in the pons).

Chronic ethanol administration

Dietary aspects. Final body weights in all experimental groups were similar in spite of the fact that

Table 1. Brain regional cell densities (CD: 10^7 cells.g^{-1} wt) and protein content (P: mg protein.g^{-1} wt) in control and chronically ethanol-treated rats

Group	Cerebral cortex		Cerebellum		Medulla		Pons		Hypothalamus		Corpus callosum	
	CD	P	CD	P	CD	P	CD	P	CD	P	CD	P
A (4)*	14.92 ± 0.73	44.24 ± 2.40	54.56 ± 1.65	51.80 ± 2.38	10.30 ± 0.26	48.77 ± 2.88	10.84 ± 0.19	52.49 ± 3.05	17.44 ± 0.68	86.01† ± 0.84	16.50 ± 0.71	74.53§ ± 3.15
B (4)	11.04† ± 0.43	37.86 ± 1.29	45.04†‡ ± 1.49	49.90 ± 2.08	8.62† ± 0.33	36.79† ± 2.88	8.88† ± 0.30	41.71† ± 2.07	11.12† ± 0.42	77.01†§ ± 0.93	13.40* ± 0.61	67.18§ ± 1.58

Values shown are means ± SE. Cell number was derived from DNA regional content by assuming a DNA content of 6.10^{-6} μg per nucleus (see Materials and Methods).

A, control rats. B, chronically ethanol-treated rats (35 days of daily intragastric administration of 4.7 g.kg^{-1} body wt of ethanol). The number of experiments is shown in brackets. Each experiment was carried out on a pool of brain tissues from 1–4 rats.

* Randomized among 10 experiments. † $P \leq 0.05$ versus control rats (Student's t-test). ‡ $P \leq 0.05$ versus all other regions (Newman-Keul's Q test). § $P \leq 0.05$ versus all other regions, except hypothalamus or corpus callosum (Newman-Keuls's Q test).

Table 2. Thiamin pyrophosphokinase activity in different brain regions from control, chronically and acutely ethanol-treated rats

Group	Cerebral cortex		Cerebellum		Medulla		Pons		Hypothalamus		Corpus callosum	
	Cell.	Prot.	Cell.	Prot.	Cell.	Prot.	Cell.	Prot.	Cell.	Prot.	Cell.	Prot.
A	5.6 ± 0.13	0.63 ± 0.009	5.3 ± 0.12	0.83 ± 0.016	6.4 ± 0.13	0.69 ± 0.031	7.3 ± 0.13	0.78 ± 0.026	6.6 ± 0.09	0.69 ± 0.016	6.4 ± 0.22	0.62 ± 0.041
B	4.9* ± 0.15	0.43* ± 0.011	3.8* ± 0.16	0.50* ± 0.017	5.1* ± 0.32	0.41* ± 0.005	5.4* ± 0.13	0.38* ± 0.017	5.3* ± 0.27	0.32* ± 0.028	4.2* ± 0.10	0.33* ± 0.017
C	4.5* ± 0.18	0.53 ± 0.027	3.1* ± 0.20	0.71 ± 0.019	5.9 ± 0.16	0.63 ± 0.014	6.7 ± 0.17	0.71 ± 0.015	5.4* ± 0.10	0.50 ± 0.011	5.8 ± 0.18	0.61 ± 0.009
D	4.7* ± 0.19	0.56 ± 0.018	3.6* ± 0.13	0.75 ± 0.022	6.1 ± 0.08	0.64 ± 0.022	6.7 ± 0.17	0.75 ± 0.019	6.0 ± 0.23	0.61 ± 0.010	5.9 ± 0.17	0.59 ± 0.024

Values shown are means ± SE of 4 experiments.

Each experiment was carried out on a pool of brain tissues from 1–4 rats.

A, control rats; B, chronically treated rats (35 days of daily intragastric administration of 4.7 g.kg^{-1} body wt. of ethanol); C and D, acutely treated rats (90 and 300 min respectively after a single intragastric dose of ethanol as above).

Cell., pmol TPP formed by 10^6 cells.hr^{-1}; Prot., nmol TPP.mg^{-1} protein.hr^{-1}.

* $P \leq 0.05$ versus controls (Dunnett's t test). Among control rats the mean TPKase activities were significantly different ($P \leq 0.05$, Newman-Keul's Q test) as follows: (a) when expressed per protein content cerebellum higher than cerebral cortex and corpus callosum only; (b) when expressed per cell number, pons higher than all others regions; cerebral cortex and cerebellum lower than all other regions.

Table 3. Effects of 35-day administration of ethanol (4.7 g kg^{-1} body wt) by gastric gavage in rats.

Group		Body weight (g)			Daily intake				Energy (kJ)	
		Initial	Final	Δ	Food (g)	Food (kJ)	Ethanol (ml)	Ethanol (kJ)	Total	Given as ethanol (%)
Control (normal)	(40)	364.4 ± 12.2	445.0 ± 9.1	+79.1 ± 7.0	32.2 ± 0.3	464.9 ± 6.4	—	—	464.9 ± 6.4	—
Ethanol* intragastric	(40)	395.5† ± 5.7	429.0 ± 6.0	+33.5† ± 5.6	26.8† ± 0.3	395.9† ± 5.0	4.8 ± 0.03	56.1 ± 0.4	451.8 ± 5.1	12.4

Values shown are means ± SE.
* 50% (v/v) ethanol solution.
The number of experiments is shown in brackets.
† $P \leq 0.05$ treated versus control (Student's *t*-test).

the body weight of controls was initially lower than that of the ethanol-treated rats (Table 3). The lower body growth of the latter was mostly due to the stress of gastric gavage, as we found in preliminary experiments using rats receiving distilled water by intragastric tube daily for 35 days. In fact, the mean growth in these rats was 41.4 ± 7.3 g (mean ± SE, N = 40) which was not significantly different ($P > 0.05$: Student's *t*-test) from the value (33.5 ± 5.6 g, N = 40) found for the chronically ethanol treated rats. Alcoholic and control rats showed a similar daily energy intake; in the former groups, 12.4% of the energy was in the form of ethanol (Table 3).

Cell density and protein content. DNA content, and hence the total number of regional cells, was lower in ethanol-treated rats than in controls (Table 1), while water content did not differ significantly between the two groups (data not shown). The reduction in total number of cells ranged from 36% in the hypothalamus to 16% in the medulla. The protein content in the different brain areas was also reduced by ethanol (Table 1), even though to a lesser extent than cellular densities. The reduction ranged from 25% in the medulla to 4% in the cerebellum, being statistically significant only for the medulla and the pons ($P \leq 0.05$: Student's *t*-test). Although chronic ethanol administration reduced regional cellular density and protein content, protein concentration per cell remained virtually unaltered in all the regions except the hypothalamus, where a 40% increase was found (data not shown). Thus hypothalamic cells displayed an increase in protein content despite a sharp decrease in number.

TPKase activity. Chronic alcoholism was associated with a statistically significant decrease in TPKase activity in all brain regions, irrespective of whether this was expressed on the basis of protein content or cell number (Table 2). In the former case the decrease ranged from 54% (hypothalamus) to 32% (cerebral cortex), while in the latter the decrease was less marked and ranged from 34% (corpus callosum) to 12% (cerebral cortex). On the whole, the cerebral cortex was the brain region where TPKase activity was least affected, while the corpus callosum was the most affected area.

Acute ethanol administration

Blood ethanol levels. After a single dose of ethanol (4.7 g.kg^{-1} body wt) by gastric gavage, peak blood alcohol levels (65.1 ± 2.8 mM, mean ± SE of 4–10 determinations, each on triplicate samples) were reached at about 60 min. These levels remained nearly unchanged for the following 90 min and thereafter decreased gradually, becoming undetectable after about 16 hr. Biochemical measurements were carried out 90 and 300 min after alcohol ingestion, when blood levels were approximately maximal (63.9 ± 3.3 mM) and half maximal (37.6 ± 0.8 mM), respectively.

Protein content. The presence of ethanol in the extracellular fluid, and hence in brain cells, did not modify protein content in the cerebral regions investigated (data not shown).

TPKase activity. A reduction in TPKase activity was observed in all brain regions at both 90 and

300 min after ethanol administration (Table 2). However, the decrease was statistically significant only when TPKase activity was expressed per number of regional cells. In this case, peak blood ethanol levels (90 min) were associated with a significant decrease in TPKase activity in the cerebellum (−42%), cerebral cortex (−20%) and hypothalamus (−18%). Lower ethanol levels (300 min) were associated with a significant decrease only in the cerebellum (−32%) and cerebral cortex (−16%). Overall, the presence of substantial amounts of ethanol in the extracellular fluid induced a marked reduction in TPKase activity only in the cells of the cerebellum and, to a lesser extent, of the cerebral cortex.

Effect of ethanol added in vitro. Addition of ethanol to the incubation medium at a wide range of concentrations, including those found in the blood of acutely treated rats, caused a dose-dependent reduction in TPKase activity in all brain regions (Fig. 1).

DISCUSSION

TPKase activity could be detected in all brain regions investigated and was highest in the cerebellum or the pons, depending on whether it was expressed on the basis of protein content or cell number respectively. Since TPKase is a completely soluble enzyme present only in the cytosol [1, 5, 26], the regional differences found are unlikely to be due to different tissue extraction. A positive relationship was apparent between total thiamin (mainly TPP) regional content [10] and TPKase activity, but only when the latter was expressed per protein content. Chronic (35 days) ethanol administration induced a marked reduction in cellular density and protein content in all regions studied, without modifying regional water content. Since the growth of chronically alcoholic rats was slower than that of controls, a non-specific influence of some nutritional deficiency cannot be excluded. The changes observed in this study are consistent with previous reports of a significant loss of certain neuronal cell types in various brain regions of rat treated chronically with ethanol [27–30]. Reduced cerebral protein content and *in vitro* biosynthesis have also been reported in alcoholic mice [31, 32]. In our study, the reduction in cell number and protein content was more prominent and diffuse than previously described. This could be explained, at least in part, by differences in experimental conditions, including type and age of animals, route, dose and duration of ethanol administration, method for evaluating cell number and, especially, type of cells considered, since our procedure based on DNA content did not discriminate between neuronal and non-neuronal (glial and endothelial) cells. On the other hand, it is well known that chronic alcoholism in man causes cerebral atrophy, as revealed by computerized tomography, suggesting that many neurons disappear along with supporting glia and vasculature [33]. The cerebellum is presently considered to be an important site of tissue damage in chronic alcoholics [34–36]. It is noteworthy that ethanol alone, independently from other factors, like malnutrition, advancing age and disease processes, may lead to irreversible cerebral damage [37].

Acute and chronic administration of ethanol, as well as the addition of ethanol to the incubation medium, induced a significant decrease in TPKase activity, although in the acute experiments *in vivo* this effect could be demonstrated only in some brain regions. These data provide the first evidence that ethanol can directly impair the phosphorylation of thiamin and thus the availability of TPP, a coenzyme vitally important for glucose metabolism in the brain. However, the specificity of the action of ethanol and the influence of the stress of gavage on TPKase remains to be clarified.

The reduction in TPKase activity may be a consequence of a general defect in the biosynthesis of proteins, although it should be noted that protein content was reduced only after chronic ethanol treatment. Alternatively, it is possible that ethanol, or its catabolite acetaldehyde (which does enter the acutely alcoholic rat brain [38], even though it is present in very small amounts [39]), can exert a direct inhibitory effect on the enzyme protein.

Interestingly, the brain regions showing a reduction in TPKase after acute alcohol intake (cerebellum, cerebral cortex, hypothalamus) are also the regions showing the greatest depression in neuronal firing activity after systemic or iontophoretic administration of ethanol [40–42].

The observation that chronic alcoholism and dietary thiamin deficiency usually cause damage to the same regions of the brain has recently led to the suggestion that the anatomical damage in alcoholism might be a consequence of a reduced metabolic ability to incorporate thiamin [43]. By increasing thiamin urinary excretion [44, 45] in rats, chronic ethanol appears to impair the ability to retain thiamin in the tissues.

According to Inokuchi *et al.* [28], dietary thiamin deficiency may enhance the degree of morphological changes induced by ethanol in cerebellar neurons. Our results show that after chronic ethanol administration the ability to phosphorylate thiamin and thus to accumulate thiamin in the tissue may be impaired in several brain regions, including cerebellum, cerebral cortex and hypothalamus. This may adversely affect cerebral metabolism and function. It is well known that glucose is the almost exclusive energy source for the brain and that chronic ethanol administration slows the glycolitic pathway and the tricarboxylic acid cycle, leading to energetic imbalance [46, 47]. Therefore, a reduction of TPKase activity, which is essential in the phosphorylation of thiamin [48], is likely to contribute to the depression of the tricarboxylic acid cycle in the tissue by reducing TPP availability. In addition, ethanol-induced inhibition of cerebral TPKase may profoundly affect the rapid turnover of regional TPP, thereby disrupting a peculiar aspect of brain thiamin metabolism and function [12].

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Note. After submission of the present paper, a short note by Matsuda *et al.* (*Experientia* **41**, 924, 1985) provided data on the regional distribution of thiamin pyrophosphokinase in normal rat brain. Even though the method of determination was different from that used in our study, the distribution of enzyme activity (expressed per mg of tissue protein) was similar to that found by us (cerebellum > medulla > hypothalamus > cerebral cortex).

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