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DECREASED ADENOSINE CYCLIC 3′,5′-MONOPHOSPHATE PHOSPHODIESTERASE ACTIVITY IN RAT BRAIN FOLLOWING TRIETHYLTIN INTOXICATION

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Abstract—The possible involvement of cerebral cAMP-phosphodiesterase (PDE) in the intoxication and brain edema formation after exposure to triethyltin (TET) has been studied *in vitro* and *in vivo* in the rat. *In vitro* studies showed an irreversible inhibition of the particulate and soluble phosphodiesterase activities. *In vivo*, both high i.v. single dose and repeated oral administration of low TET doses led to a significant decrease of the particulate activities. Phosphodiesterase inhibition preceeded edema formation. The soluble activities were less influenced and their inhibition could be a consequence of the edema formed in the brain tissue rather than of a TET direct action upon the enzyme. Kinetic analysis of the brain particulate enzyme from the TET-treated rats showed a significant decrease in the V_{max} of the substrate high affinity enzyme form when compared to controls.

Triethyltin (TET), a highly neurotoxicant agent, appears to be a suitable model substance for inducing experimental cytotoxic brain edema [1, 2]. This pathological condition is characterized by abnormal accumulation of water and sodium in glial, neuronal and endothelial cells from brain tissue and by a marked histotoxic hypoxia [2]. When severe, brain edema may be responsible for generalized signs of brain dysfunction such as decreases in spontaneous motor activity, deficits in maze performance, in acoustic startle responses and in visually evoked reflexes [1, 3, 4].

The mechanism of brain edema generation through TET poisoning remains largely unknown. TET induces various perturbations in glucose [5] and energy [6] metabolism which may reflect the injury of cell membranes occurring in brain edema. Besides, the activity of several membrane-linked enzymes such as mitochondrial Mg^{2+} ATPases, Na–K ATPases or 5′-nucleotidase are modified *in vitro* by TET [7]. On the other hand, cyclic AMP, known to regulate the transport of water and electrolytes in various cells [8], could participate in the genesis and/or development of brain edema. Indeed, several studies have shown that cyclic AMP accumulates to concentrations 2–20-fold over basal levels in the anoxic brain [9, 10]. However, reports concerning the effect of triethyltin on the enzymes related to the cylic AMP systems are few.

Thus, Leow *et al.* [11] showed that TET inhibits basal adenylate cyclase activity of brain homogenate *in vitro* but not *in vivo*.

Siebenlist and Taketa pointed out the dual complex effect of TET on protein kinases [12, 13] comprising at first an enzyme activation due to the TET binding to the regulatory subunits of the enzyme and releasing of the active catalytic subunits followed by a slow, apparently irreversible inactivation due to a subsequent TET binding now to the released catalytic subunits.

A single report [7] is related to the influence of TET upon cyclic nucleotide phosphodiesterase (PDE) (EC 3.1.4.17). In this study Wassenaar and Kroon [7] observed a 47% *in vitro* inhibition of a particulate PDE fraction from rabbit brain, measured with high substrate concentration and in the presence of 10^{-4} M TET.

Cyclic AMP-phosphodiesterase, present in high level within brain tissue and especially in synaptosomal fraction [14], plays a crucial role in regulating cyclic AMP level, as emphasized by theoretical analyses [15, 16]. Therefore, we thought it was of interest to investigate further the influence *in vitro* and *in vivo* of TET upon brain cyclic AMP-phosphodiesterase.

In the present work, we examine the effect of repeated orally administered and of a single i.v. injection of TET upon the different forms of phosphodiesterase activity in rat brain. Additional *in vitro* experiments are also reported.

MATERIALS AND METHODS

Male Sprague–Dawley rats aged 6–8 weeks and weighing about 180–240 g were caged under standard cycling conditions and fed a standard diet of pellets and water *ad libitum*.

Triethyltin intoxication. This was carried out by i.v. and by oral administration of triethyltin sulphate (Merck). In the i.v. administration a single dose of 10 mg/kg of TET sulphate in NaCl 0.15 M was injected to the treated group while controls received the vehicle. For the oral administration, treated groups were exposed for 4, 10 and 15 days to triethyltin sulphate 20 mg/l, dissolved in their drinking water. Edema formation in the brain tissue in the

case of i.v. intoxication was checked by determinations of the specific gravity in cerebral cortex and white matter using a continuous Percoll density gradient, according to the technique proposed by Tengvar *et al.* [17].

Soluble and particulate PDE preparation. Crude cytosolic and particulate cyclic nucleotide phosphodiesterases from control and TET intoxicated rat brain were prepared as previously described [18]. Cytosolic (1 hr, 105,000 *g* supernatant) and particulate (1 hr, 105,000 *g* pellet) preparations were freshly used, after appropriate dilutions in 40 mM Tris–HCl buffer pH 8.0, for enzyme activity assays and kinetic studies. For the *in vitro* studies, frozen and thawed preparations were used.

Phosphodiesterase assay. Enzyme activity was assayed by the two step radio-isotopic procedure of Thompson and Appleman [19], modified as described in [20]. The enzyme forms with high affinity for cyclic AMP (low K_m activities) and with low affinity for cyclic AMP (high K_m activities) were measured with 0.25 and 50 μM cyclic AMP concentrations respectively. Kinetic studies were carried out for a cyclic AMP concentration range of 0.25–250 μM. In the experimental conditions used, enzyme activity was linear with respect to time and enzyme concentrations.

As triethyltin induces important changes in the water content of brain tissue, cyclic AMP-phosphodiesterase activity was expressed as nmoles or pmoles cyclic AMP hydrolysed per min and per mg fresh tissue, mg proteins and μg DNA. Results were examined comparatively for all three cases.

Protein was assayed by the Lowry method with bovine serum albumin as standard. DNA was measured according to the fluorometric method of Karsten and Wollenberger [21].

The means ± S.E.M. were compared with a two way analysis of variance, P = 0.05 being taken as the limit of statistical significance.

Kinetic data were fitted to different models: Michaelian model, sum of two Michaelian enzymes, Hill model, by a non-linear least squares computer program as performed in [20].

RESULTS AND DISCUSSION

In vitro *experiments.* When added directly to the assay medium, in concentrations as high as 10^{-4} M, TET did not influence the activity of the particulate or soluble forms of the enzyme. On the contrary, when TET and the enzyme were preincubated together at pH 8.0 and 30° before enzyme activity was assayed, an inactivation was observed which progressed with time and TET concentrations. Thus, after 30 min of preincubation in the presence of 10^{-4} M TET, the particulate phosphodiesterase presented a significant loss of enzymatic activity (33%) for the low K_m (Fig. 1A) and high K_m forms (not shown). For the soluble cAMP phosphodiesterase, the inhibition was evident starting with 5 min of preincubation in the presence of TET 10^{-4} M and reached 30% for the high K_m and 40% for the low K_m activities after 20 min (not shown). In view of these results the *in vitro* inactivation of brain PDE by triethyltin, increasing with time, is of the irreversible

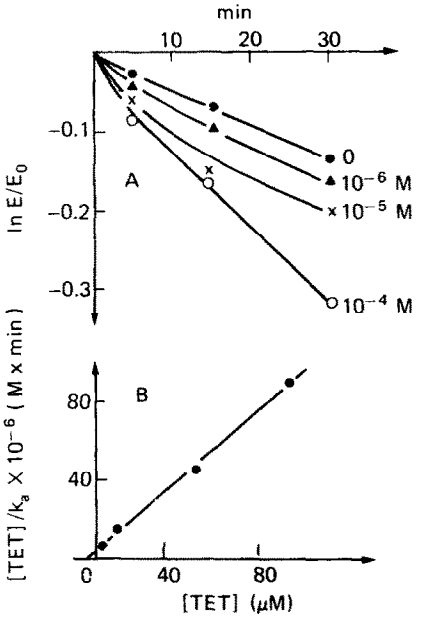

Fig. 1. Time course of rat brain low K_m particulate cyclic AMP phosphodiesterase inactivation by triethyltin *in vitro*. Frozen and thawed 105,000 pellet was incubated with indicated concentrations of TET at 30° in 40 mM Tris–HCl buffer pH 8.0 A. At the indicated times, an aliquot was removed and assayed as described under Materials and Methods. Enzyme activity was measured in triplicate for substrate concentration of 0.25 μM. E_o: Enzyme activity in the absence of TET; E: enzyme activity incubated with TET. B = determination of k_2 and K for the interaction of the particulate low K_m enzyme with triethyltin. The value of K_m = 2.5 μM was obtained from independent experiments.

type. Pseudo-first order rate constants for the inactivation were computed from plots of ln (E/E_o) against t, where E_o and E were the enzyme activities at zero time and at time t respectively.

If the general model for the interaction between enzyme, inhibitor and substrate in non-steady state situations proposed by Frere *et al.* [22] is considered and the simplifying case of a competitive interaction between substrate and inhibitor is assumed, apparent rate constants for the formation of the inactive complex EI^+

$$k_a = \frac{k_2}{1 + \frac{K}{[I]}\left(1 + \frac{[S]}{K_m}\right)}$$

are measured, where K and K_m are affinity constants for TET and cAMP respectively, [S] is the concentration of cAMP and k_2 characterizes the velocity of the inactivation process.

The graph of $[I]/k_a$ against [I] yields a line of slope $1/k_2$ and an intercept:

$$\frac{K\left(1 + \frac{[S]}{K_m}\right)}{k_2}$$

allowing one to calculate k_2 and K. In the case of the particulate phosphodiesterase, K and k_2 for the low K_m enzyme form were 3.2×10^{-6} M and 70 sec^{-1}

respectively (see Fig. 1B). For the high K_m enzyme the values of K and k_2 were 2.4×10^{-6} M and 116 sec^{-1} respectively.

At least two hypotheses might be made in order to explain the fact that the particulate activity was significantly inactivated after 30 min of preincubation with TET while the soluble forms were modified as soon as after 5 min: either soluble and particulate enzyme forms, due to the differences in their structures, are reacting differently against TET inactivation; or the inactivation is influenced by the chemical environment of the enzyme, the particulate enzyme, tightly bound to the membranes, being less accessible or more stable than the soluble one towards TET attack *in vitro*.

In vivo *studies*. Taking into account the first results obtained *in vitro*, it seemed interesting to investigate the modification of brain PDE activity *in vivo* after TET intoxication.

Repeated oral administration of low TET doses represents a convenient and not aggressive technique, from the point of view of mortality, in obtaining experimental cytotoxic brain edema. In this kind of treatment, the daily doses of TET absorbed are very low (1.3 mg TET/kg as approximated from the quantity of water drunk by the animals). According to the neuropathological results reported in the literature [23, 24], brain tissue is altered as soon as day 4–5, with intramyelinic vacuoles in the central white matter being evident, and with edema fully developed by the 15th day.

After 15 days of intoxication, we noted an increase in the brain weight of 12% in the treated rats as compared to the same age control group; these results are in good agreement with those of Wassenaar and Kroon [7] obtained with rabbit brain after repeated i.p. administration of low TET doses.

In response to the 15-day TET administration, cyclic AMP-phosphodiesterase activity in the rat brain showed a significant decrease in both "high K_m" and "low K_m" particulate activities when compared to controls (Table 1). This decrease appears to be important (33 and 21% for the high and low K_m activities respectively when results are expressed as pmoles/min/mg fresh tissue) but in this case results are likely to be overestimated on account of water accumulation in the edematous brain. On the other hand, results expressed as pmoles/min/mg proteins might be underestimated since some data from the literature indicate a partial deproteinization of myelin during edema [25]. In contrast, DNA content of the tissue, which can be a measure of cell number was not reported to vary during TET treatment. We, too, did not find any significant difference concerning brain DNA content between control (1638.5 ± 93.0 μg/brain) and TET treated (1686.0 ± 67.0 μg/brain) rats of the same age. Results expressed as pmoles/min/μg DNA pointed out a still significant decrease (23 and 19%) in the particulate PDE activity of the TET intoxicated rat brain (Table 1).

In the case of the soluble phosphodiesterase, the high K_m and the low K_m forms showed significant inhibition only when the enzyme activity was given as a function of fresh tissue wt (21%) or of DNA content of the tissue (24 and 18% respectively) (Table 1).

In another series of experiments, we have investigated the changes in the phosphodiesterase activity as a function of the time the orally TET intoxication was carried out (Fig. 2). The low K_m phosphodiesterase, soluble or particulate, presented a continuous loss of activity which became significant, if compared to the normal group, on days 15 and 10 of the intoxication period, respectively. The high K_m enzyme forms were highly influenced starting with day 4 of the treatment (48% of inhibition for the soluble form and 38% for the particulate one), the activity recovering afterwards but remaining, on day

Table 1. 3′,5′Cyclic AMP phosphodiesterase activity in brain from controls and triethyltin treated rats

		Particulate 3′,5′-cAMP phosphodiesterase (105,000 *g* pellet)					
		High K_m			Low K_m		
		(nmol/min/mg tiss.)	(nmol/min/mg prot.)	(pmol/min/μg DNA)	(pmol/min/mg tiss.)	(nmol/min/mg prot.)	(pmol/min/μg DNA)
Control	(9)†	0.52 ± 0.09	8.4 ± 0.6	420 ± 60	9.8 ± 0.7	0.18 ± 0.01	8.2 ± 0.3
TET	(9)†	0.35 ± 0.06	7.1 ± 1.0	352 ± 76	7.7 ± 1.5	0.15 ± 0.02	6.7 ± 1.1
Inhib. %	‡	33	15	23	21	14	19
		Soluble 3′,5′-cAMP phosphodiesterase (105,000 *g* supernatant)					
		High K_m			Low K_m		
		(nmol/min/mg tiss.)	(nmol/min/mg prot.)	(pmol/min/μg DNA*)	(pmol/min/mg tiss.)	(nmol/min/mg prot.)	(pmol/min/μg DNA*)
Control	(9)†	0.95 ± 0.12	31.9 ± 4.3	864 ± 123	15.4 ± 1.9	0.50 ± 0.10	12.7 ± 1.0
TET	(9)†	0.76 ± 0.12	29.2 ± 4.1	660 ± 96	12.1 ± 2.3	0.45 ± 0.13	10.4 ± 1.0
Inhib. %	‡	21		24	21		18

The enzyme activity was measured in triplicate with substrate concentration of 50 μM for the high K_m forms and 0.25 μM for the low K_m forms. Mean values ± S.E.M. are given.

* Activity was expressed in terms of brain total DNA.

† Number of animals studied.

‡ Percentage inhibition was indicated only when significant difference between treated and control groups for $P < 0.05$ was found using variance analysis test.

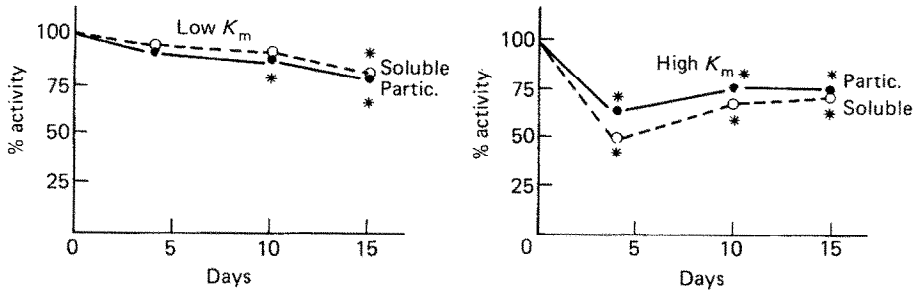

Fig. 2. Cyclic AMP phosphodiesterase activity in the rat brain as a function of the intoxication time period with TET in the drinking water. *Significant difference with control values for $P < 0.05$.

15, still significantly lower when compared to controls (Fig. 2) (24 and 23%).

In order to further investigate the influence of TET intoxication on the particulate PDE activity, kinetic studies of cyclic AMP hydrolysis were carried out over a wide range of cyclic AMP concentrations in both control and 15 days-TET treated rats. Lineweaver–Burk representation gave concave downward plots (Fig. 3) in control as well as in treated group. Two distinct apparent K_m and V_{max} values could be graphically obtained by linear extrapolation (Table 2). Among the three kinetic models tested by the computer aided curve-fitting procedure, as described in Materials and Methods, the best-fitting was obtained for the two Michaelis kinetics model. As shown in Fig. 3 and Table 2, TET administration affected only the kinetics of the particulate "low K_m" activity, significantly decreasing its V_{max} value, without modification of the apparent K_m. The "Hill kinetics" model fitting also showed a modification in the V_{max} value of the particulate PDE activity after TET intoxication with no significant change in the apparent cooperativity (Hill number not modified) (Table 2).

The inactivation of PDE activity *in vivo* (about 19–23%) after repeated oral administration of low doses of TET appears to be rather low. But, it becomes important if one takes into account the specific character of phosphodiesterase action which is represented by the sum of two enzyme activities "low and high K_m forms", governing cAMP degradation and exhibiting negative co-operativity. Erneux *et al.* [16] showed that such kinetics ensure an enhanced sensitivity of cAMP accumulation even to slight decreases in PDE maximal velocity and, by this way, make this enzyme a highly efficient system for regulating cAMP concentration in the cell. Furthermore, Fell [15], by means of a theoretical compartmented model, concluded that in brain tissue, due to the high concentration of cAMP in the vicinity of the membranes, the level of cAMP in the cell has to be very sensitive to modulation of the particulate enzyme.

The question is whether such an inhibition found *in vivo*, after 15 days of TET intoxication is due to the direct action of triethyltin upon the brain PDE or to the development of brain cytotoxic edema [2] which may also influence the PDE activity, indirectly, through the general fall in the energy metabolism [5, 6, 11].

In order to examine this problem, we have investigated the changes in the PDE-activity in parallel with edema development after a single intravenous dose of 10 mg TET/kg. At this dose, the con-

Table 2. Kinetic parameters of the particulate cyclic AMP phosphodiesterase activity from control and triethyltin treated rat brain, determined by linear regression analysis and by computer fitting to "2 Michaelis kinetics" and to "Hill kinetics" models

		"2 Michaelis" model			
		High K_m		Low K_m	
		V_{max} (pmol/min/μg DNA)	K_m (μM)	V_{max} (pmol/min/μg DNA)	K_m (μM)
Regression analysis	Control	1302 ± 64	138 ± 13	37.6 ± 2.6	0.64 ± 0.06
	TET	1007 ± 143	117 ± 15	29.0 ± 2.5	0.53 ± 0.04
Computer fitting model	Control	1347 ± 71	159 ± 12	18.4 ± 1.1	0.33 ± 0.03
	TET	1240 ± 85	176 ± 17	15.3 ± 0.1	0.29 ± 0.03

		"Hill" model		
		V_{max} (pmol/min/μg DNA)	K_m (μM)	Hill number
Computer fitting model	Control	2203 ± 209	127 ± 11	0.80 ± 0.03
	TET	1666 ± 220	123 ± 9	0.83 ± 0.03

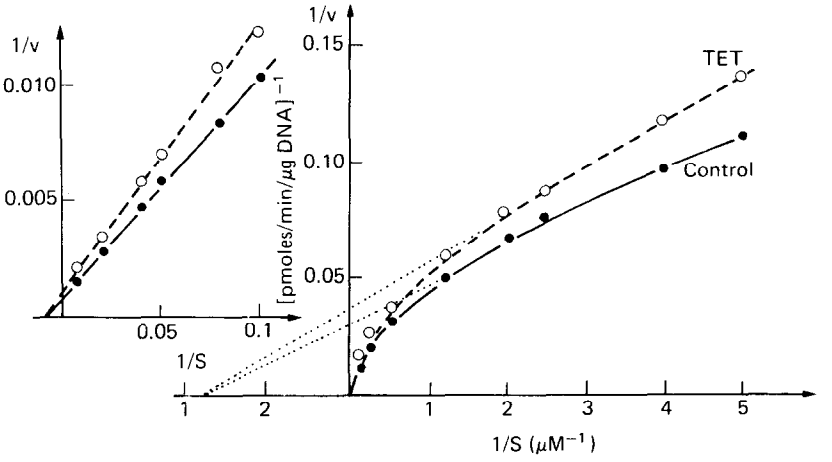

Fig. 3. Lineweaver and Burk plots obtained with particulate phosphodiesterase fraction from control and TET intoxicated rat brain. Control ●——●. TET intoxicated rat O---O. Cyclic AMP phosphodiesterase activity was measured in triplicate with substrate concentration ranging from 0.25 to 250 μM. The inset shows the same data on an expanded scale. For assay conditions see Materials and Methods.

centration of TET in the brain increases over the first hour up to 20 μM and then remains stable for at least 48 hr [13, 26] with no gross differences in the distribution of TET throughout the brain [7, 26, 27]. On the other hand, as shown by Tengvar *et al.* [17] by means of electron microscopy studies and determinations of specific gravity of brain tissue in mice, edema begins to develop in mice white matter, 4 hr after the 10 mg TET/kg single i.p. dose has been administered and is fully developed after 24 hr.

For the rat brain too, we noted no difference in mean density values between TET treated animals and controls, 1 hr after the i.v. injection, in samples from both white and grey matter. In contrast, 24 hr after the i.v. injection, there are significant decreases in density values, the difference being 0.0035 for the cerebral cortex and 0.0079 for white matter and indicating the development of brain edema (Fig. 4).

As shown in Fig. 4, in brains from rats killed at 1 hr following an i.v. injection of 10 mg TET/kg, the particulate phosphodiesterase activity is significantly lowered when compared to controls: 18 and 25% for the low K_m and high K_m activities, respectively. In the brain of rats allowed to survive 24 hr after the i.v. injection, the low K_m form continues to decrease, reaching 31% of inhibition while the high K_m activity is not further diminished, but seems even to recover slightly (19% inhibition after 24 hr). The results obtained demonstrate that *in vivo*, TET in a concentration of 10–20 μM acts directly on the particulate phosphodiesterase inhibiting both high K_m and low K_m activities before edema formation. When an edema is formed in the brain tissue, 24 hr after the i.v. injection, an enhanced inhibition of the particulate low K_m form can be observed. This well marked inhibition of the low K_m PDE form was in good agreement with the kinetic studies on the particulate enzyme from control and 15 days intoxicated rat, where major modifications appeared in the kinetics of the low K_m enzyme.

After the i.v. administration of TET, the soluble PDE activity was found modified only for the low K_m form and only 24 hr after the injection.

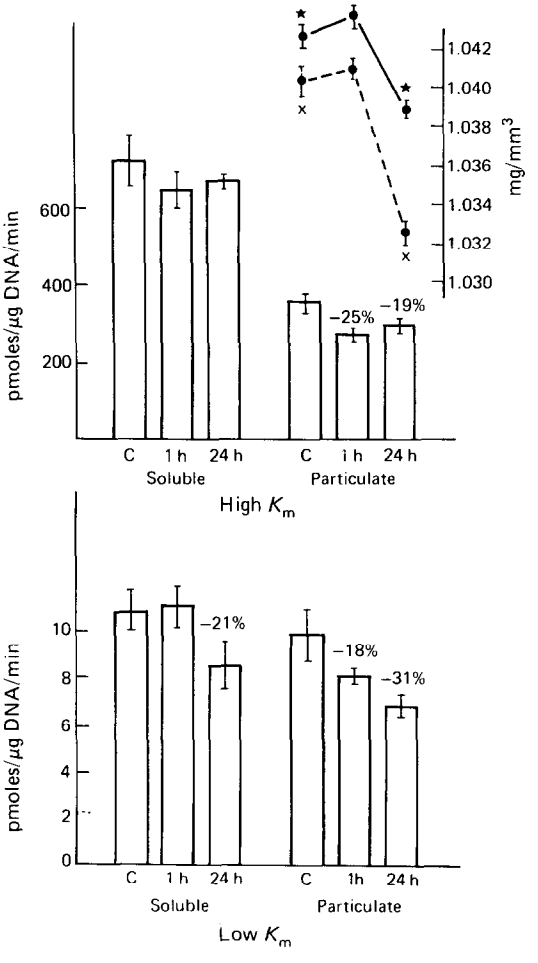

Fig. 4. Density values and cyclic AMP phosphodiesterase activity in the brain of normal rats and following a single i.v. dose of 100 mg TET. Densities were measured in Percoll (300 mOsm/l) gradients in the density range 1.0200–1.0600 g/ml. Means ± S.D. are given for 5–6 animals tested. ★, × marks significant difference for $P < 0.05$. Grey matter ●——●. White matter ●---●.

Table 3. The ratio soluble/particulate cAMP phosphodiesterase activity in the brain of control rats and following TET intoxication

Administration		Soluble activity / Particulate activity	
		Low K_m	High K_m
i.v. (6)	N	0.75 ± 0.04	1.31 ± 0.10
	1 hr	0.93 ± 0.09*	1.61 ± 0.14*
	24 hr	0.76 ± 0.09	1.36 ± 0.01
Oral (8)	N	0.93 ± 0.14	1.45 ± 0.22
	4 days	0.94 ± 0.11	1.25 ± 0.22
	10 days	0.93 ± 0.11	1.52 ± 0.23
	15 days	0.96 ± 0.10	1.30 ± 0.21

The number of animals studied is given in parentheses.
* Indicates significant difference between treated and control groups.

One explanation for these results can be a possible accumulation of TET in the membrane phospholipids due to its fat solubility; this may result in a higher concentration of the drug in the particulate enzyme neighbourhood than in the cytoplasm, leading thus to an earlier observed effect on the particulate rather than on the soluble enzyme forms. Nevertheless, other explanations must not be neglected as in experiments concerning the time-course of the orally TET intoxication we obtained an important inhibition of the soluble activity for low doses of TET but as soon as an edema was formed in the brain tissue (Fig. 2). Therefore, the decrease in the soluble activities can be also a consequence of the cytotoxic condition and edema formed in the brain tissue or a result of some other mechanism as a compensatory re-repartition of PDE activity between soluble and particulate compartment of the cell. This last supposition is suggested by the values of the ratio soluble/particulate activity for the two ways of TET administration studied and given in Table 3. It can be seen that administration of a single dose of TET produces at first an important increase in this ratio which comes back to normal 24 hr later while the repeated administration of low TET doses does not modify this ratio, even after an edema is formed.

In conclusion, our studies show that the different forms of the cerebral PDE are sensitive *in vivo* towards TET inhibiting activity. Results obtained with the i.v. treatment, indicating that PDE inhibition preceeds edema formation allow to suppose that the implication of PDE inhibition in TET intoxication and in the process of edema formation has not to be excluded; further studies aiming to prove this hypothesis are necessary. Besides, as TET action may imply a general conformational change in membrane structures or in the structure of some lipid components known to stabilize and/or activate phosphodiesterase molecules, information in this field should be useful in understanding the mechanism of TET inhibition of the enzyme and perhaps, its relationship to cellular cytotoxic condition.

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