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THIOPENTAL INHIBITION OF γ-AMINOBUTYRATE TRANSAMINASE IN RAT BRAIN SYNAPTOSOMES

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Abstract—The kinetic parameters for thiopental inhibition of γ-aminobutyrate: 2-oxoglutarate transaminase solubilized from rat brain synaptosomes were studied. V_{max} is 0.76 μmole/hr/mg of protein. K_m for GABA (γ-aminobutyric acid) is 16.0 mM and K_m for 2-OG (2-oxoglutarate: α-ketoglutarate) is 0.35 mM. Thiopental inhibition is noncompetitive and K_i, 1.1 mM, is similar when either GABA or 2-OG is the limiting substrate. The implication of these findings in clinical anesthesia is discussed.

Ransom and Barker [1] have recently summarized physiological evidence for a GABA (γ-aminobutyric acid) mechanism of action for pentobarbital. They further demonstrated that pentobarbital at anesthetic concentrations enhanced post-synaptic inhibition produced by GABA but not by glycine. Additional pharmacological studies suggested the participation of GABA in pentobarbital anesthesia, but no clear mechanism emerged. In one study [2], pentobarbital increased brain GABA content and reduced GAD* activity. In another study [3], pentobarbital did not alter brain GABA content or GAD activity, but the rates for [^{3}H-]GABA disappearance were decreased by pentobarbital. Acute pentobarbital administration reduced the fast component and chronic administration reduced the slow component of GABA disappearance. GABA-T activity was unaltered.

We have hypothesized that anesthetic agents exert their action through interaction with GABA metabolism [4, 5]. A reduction in GABA catabolism was suggested as a basis for an observed increase in GABA content in brain slices. Using a synaptosomal system to study GABA catabolism, we found that several anesthetic agents, including the barbiturates, inhibited the liberation of $^{14}CO_2$ from [1-^{14}C]GABA in intact synaptosomes [6]. An inhibition by barbiturates of GABA-T activity solubilized from synaptosomes was also demonstrated [7]. This communication describes the inhibition of solubilized GABA-T activity by thiopental. These findings are compatible with a mechanism of barbiturate action which involves GABA-induced synaptic inhibition.

MATERIAL AND METHODS

Rat forebrain synaptosomes were prepared according to the sucrose-density centrifugation method [8]. The synaptosomes were finally suspended in a medium containing Tris 100 mM, mannitol 450 mM, sucrose 150 mM, KH_2PO_4 5 mM, NaH_2PO_4 5 mM, disodium EDTA 0.1 mM, and Na-succinate 10 mM, adjusted to pH 7.4 with HCl. These preparations can be kept frozen without significant loss in GABA-T activity, while the enzyme activity in synaptosomes frozen in 0.32 M sucrose decreased rapidly. Several batches of synaptosomes were pooled and used in these experiments. In the final assay mixture, Triton X-100 was added to insure the dissolution of synaptosomes. Their protein contents were determined using phenol reagent [9].

Assay of solubilized GABA-T activity was carried out at 30° according to methods described previously [10, 11]. The complete system in 1.0 ml contained Tris 50 mM, Triton X-100 0.5%, dithiothreitol 1 mM, pyridoxal phosphate 0.4 mM, GABA 75 mM, 2-OG (2-oxoglutarate; α-ketoglutarate) 5 mM, aldehyde dehydrogenase 0.25 units/ml, NAD 8.3 mM, NADH 17 μM, synaptosomes and inhibitor. All the components except the limiting substrate and thiopental were mixed and preincubated for a minimum of 1 hr. Either GABA or 2-OG was added to initiate the reaction and absorbancy at 340 nm was read at 5-min intervals for 45 min. The kinetic profile of the reaction (see Fig. 1) shows an initial lag of approximately 5 min, followed by a gradual increase in the reaction rate as incubation continued. This inital lag was characteristic in coupled enzymic reactions where the product was being determined. The hourly rate was calculated from the absorbancy differences between 10 and 40 min after the initiation of the reaction, since the reaction rate in this period was approximately linear with time. This rate was converted to μmoles/hr. Both preincubation and the inclusion of a small amount of NADH reduced the initial dip in absorbancy and helped to attain the maximal reaction rate of that synaptosomal preparation.

Thiopental was added after preincubation before the initiation of reaction with the limiting substrate. It could not be added at more than 2 mM in this assay system since precipitation occurred in the cuvette.

Specifically, five sets of experiments, each at a different thiopental concentration, were performed in order to plot these data kinetically to calculate K_m, V_{max} and K_i. In each set of experiments with a fixed thiopental concentration, the substrate concentration

* Enzymes: GAD: EC 4.1.1.5 – glutamate decarboxylase, L-glutamate 1-carboxylyase; aldehyde dehydrogenase: EC 1.2.1.5 – aldehyde: NAD(P)$^+$ oxidoreductase; GABA-T: EC 2.6.1.19 – GABA transaminase, aminobutyrate aminotransferase, 4-aminobutyrate: 2-oxoglutarate aminotransferase, γ-aminobutyrate: 2-oxoglutarate transaminase; and GDH: EC 1.4.1.2 – glutamate dehydrogenase, L-glutamate: NAD$^+$ oxidoreductase (deaminating).

Table 1. Effect of varied amounts of synaptosomes on GABA-T activity*

Synaptosomes	ΔA hr
Expt. A: 5 mg protein/ml	
25 μl	0.316
50 μl	0.648
75 μl	1.226
Expt. B: 3 mg protein/ml	
25 μl	0.230
50 μl	0.554
75 μl	0.916

* Enzyme activities are 0.47 and 0.60 μmole/hr × mg of protein in Expt. A and B respectively. See text for experimental details.

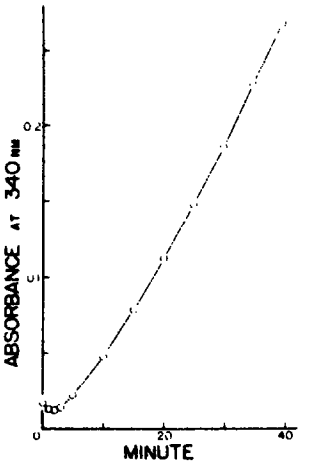

Fig. 1. Time course of GABA-T reaction. An aliquot (0.05 ml) of a synaptosomal suspension containing 2.4 mg protein/ml was employed. For assay conditions, see text.

was varied and pairs of experiments were performed with and without added thiopental. A third cuvette contained sufficient substrates so that the maximal reaction rate was limited by the amount of synaptosomes (i.e. enzyme). This cuvette served to safeguard against the use of enzyme preparation which had lost substantial activity.

Within each set of experiments, four kinetic plots were utilized to describe the data. They were a Lineweaver–Burk plot, a Hanes–Woolf plot, a Woolf–Augustinsson–Hofstee plot and an Eadie–Scatchard plot. In each plot the slopes, X- and Y- intercepts from regression lines describing the control and thiopental-inhibited data, were put through conventional two-tailed *t*-tests. The results from these kinetic analyses served to determine the nature (or type) of inhibition. The multiple kinetic analyses were undertaken because the data did not always fit straight lines exactly in any single plot. Since each datum point weighed differently in calculating the regression lines by different plots, averaging the resultant K_m values served to average out these uneven weights and gave a better measure of the true K_m.

After completing five sets of experiments at different thiopental concentrations, the K_m values from these data sets with the same limiting substrate were compared statistically. The K_m values were found to be similar and, therefore, were averaged to give a single statistical average which represents the best estimated K_m for that substrate. This value was used to calculate a regression line which passed through this point and fit the original data. Only Lineweaver–Burk plots are given in Results, not Hanes–Woolf, Woolf–Augustinsson–Hofstee and Eadie–Scatchard plots. The V_{max} values were calculated from pooled control data.

Every substrate concentration was represented in each set of experiments with a fixed thiopental concentration. These data, both control and thiopental inhibited, were used to calculate K_i according to the Dixon plot. A statistical technique similar to that described in the preceding paragraph was utilized to arrive at a best estimate of K_i.

Table 2. Effect of thiopental on GABA-T reaction rates with varying concentrations of GABA*

		[S]							
[I]		5	7.5	10	15	25	50	K_m	V_{max}
0.382	C	0.241	0.268	0.336	0.421	0.528	0.646	12.9	0.785
	I	0.196	0.235	0.281	0.354	0.452	0.541	13.2	0.671
0.573	C	0.208	0.286	0.352	0.440	0.554	0.648	15.5	0.878
	I	0.149	0.204	0.248	0.310	0.288	0.449	14.5	0.598
0.764	C	0.186	0.294	0.333	0.456	0.540	0.644	18.0	0.925
	I	0.115	0.199	0.206	0.309	0.356	0.433	21.4	0.653
0.955	C	0.187	0.253	0.336	0.401	0.514	0.590	16.1	0.818
	I	0.086	0.126	0.198	0.207	0.288	0.323	22.9	0.514
1.146	C	0.220	0.284	0.369	0.418	0.511	0.594	11.6	0.745
	I	0.104	0.147	0.195	0.212	0.276	0.317	14.2	0.423
Pooled	C	0.209	0.277	0.345	0.427	0.530	0.624	14.4	0.824
K_i		0.69	0.88	1.30	0.95	1.18	1.07		

* S = GABA; I = thiopental and C = control. [S], [I], K_m and K_i are given in mM. Reaction rates are given in μmoles/hr × mg of protein. K_m and V_{max} are averages from four kinetic plots (Lineweaver-Burk, Hanes-Woolf, Woolf–Augustinsson–Hofstee and Eadie–Scatchard). The statistical average of K_m values from all five C and I experiments is 16.0 ± 1.2 mM, and V_{max} from five controls only is 0.830 ± 0.032 μmole/hr × mg of protein. Average K_i is 1.01 ± 0.09 mM.

RESULTS

The time course of GABA-T reaction under these conditions was not linear but was very reproducible (Fig. 1) when the difference in absorbancy at 340 nm between 10 and 40 min after the initiation of the reaction was taken as a measure to calculate the hourly rate in μmoles GABA or 2-OG reacted. The rate was proportional to the amount of synaptosomes added (Table 1). Yeast aldehyde dehydrogenase, although added, was not necessary, since there was enough endogenous oxidation of succinic semialdehyde so that this portion of reaction was not rate limiting in the coupled assay. Thiopental did not inhibit this portion of the coupled assay.

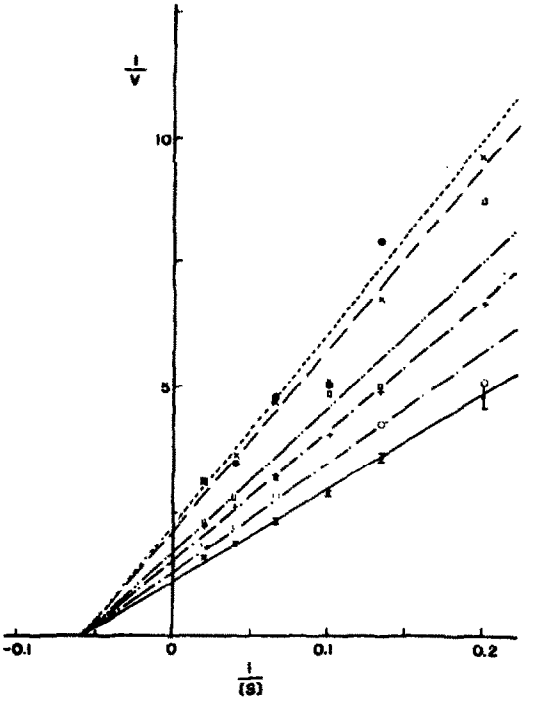

Fig. 2. Lineweaver–Burk plot of data in Table 2. S = GABA in mM, I = thiopental in mM, and V = μmoles/hr × mg of protein. Different regression lines plotted are controls (mean ± S. E. M.; ——————), 0.382 mM I (○; ——.·.·.——.), 0.573 mM I (+; ——.—.—.—.), 0.764 mM I (□; —..—..—..), 0.955 mM I (●; - - - - - - - -), and 1.146 mM I (×; — — —).

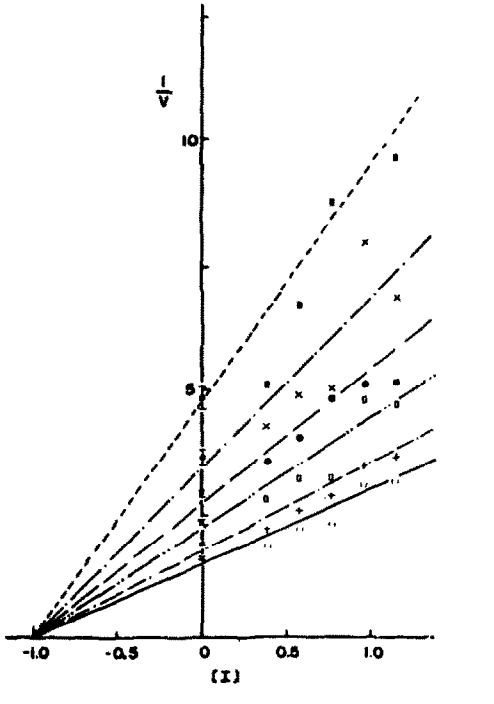

Fig. 3. Dixon plot of data in Table 2. S = GABA in mM, I = thiopental in mM, and V = μmoles/hr × mg of protein. Different regression lines plotted are 5 mM S (■; - - - - - - - -), 7.5 mM S (×; —.—.·.·.—.), 10 mM S (●; — — — —), 15 mM S (□; —..—..—.. —), 25 mM S (+; — —. —.) and 50 mM S (○; ——————).

Table 3. Effect of thiopental on GABA-T reaction rates with varying concentrations of 2-OG*

		[S]							
[I]		0.1	0.15	0.2	0.3	0.5	1.0	K_m	V_{max}
0.191	C	0.153	0.207	0.266	0.353	0.423	0.524	0.374	0.744
	I	0.143	0.197	0.233	0.312	0.392	0.461	0.338	0.639
0.382	C	0.150	0.200	0.252	0.329	0.402	0.461	0.312	0.635
	I	0.117	0.169	0.200	0.259	0.315	0.345	0.278	0.472
0.573	C	0.156	0.205	0.228	0.306	0.397	0.497	0.314	0.624
	I	0.099	0.146	0.153	0.231	0.277	0.316	0.331	0.442
0.764	C	0.136	0.213	0.242	0.305	0.388	0.463	0.344	0.643
	I	0.087	0.118	0.169	0.186	0.242	0.312	0.386	0.435
0.955	C	0.132	0.193	0.245	0.333	0.414	0.494	0.466	0.782
	I	0.094	0.106	0.160	0.175	0.231	0.289	0.326	0.376
Pooled	C	0.146	0.204	0.247	0.325	0.405	0.488	0.362	0.686
K_i		1.22	0.99	1.29	1.03	1.16	1.20		

* S = 2-OG; I = thiopental; and C = control. [S], [I], K_m and K_i are given in mM. Reaction rates are given in μmoles/hr × mg of protein. K_m and V_{max} are averages from four kinetic plots (Lineweaver–Burk, Hanes–Woolf, Woolf Augustinsson–Hofstee and Eadie–Scatchard). The statistical average for K_m is 0.347 ± 0.011 mM, for V_{max} is 0.686 ± 0.019 μmole/hr × mg of protein, and for K_i is 1.15 ± 0.05 mM.

The optimal concentration of 2-OG was determined experimentally; the maximum rate was obtained at approximately 5 mM 2-OG. High concentrations of 2-OG caused inhibition of GABA-T. When glutamate was substituted for GABA no reaction occurred, indicating that no significant amount of GDH was present to interfere with the assay system.

Where GABA was the rate-limiting substrate, the results (Table 2) showed that the K_m was not altered statistically by the presence of thiopental. The K_m for pooled data was 16.0 ± 1.2 mM and the V_{max} for experiments performed in the absence of thiopental was 0.83 ± 0.03 μmole/hr/mg of protein. All four kinetic plots showed noncompetitive inhibition by thiopental. Only the Lineweaver Burk plot (Fig. 2) is shown. The Dixon plot (Fig. 3) shows a K_i of 1.01 ± 0.09 mM.

In a similar manner, the results with 2-OG as a limiting substrate (Table 3) showed a K_m of 0.35 ± 0.02 mM, a V_{max} for controls of 0.69 ± 0.03 μmole/hr/mg of protein, and a K_i of 1.15 ± 0.05 mM. The K_i values under both GABA or 2-OG limiting conditions were similar statistically and were averaged to 1.1 mM. The V_{max} values in both sets of control experiments were averaged to 0.76 μmole/hr/mg of protein.

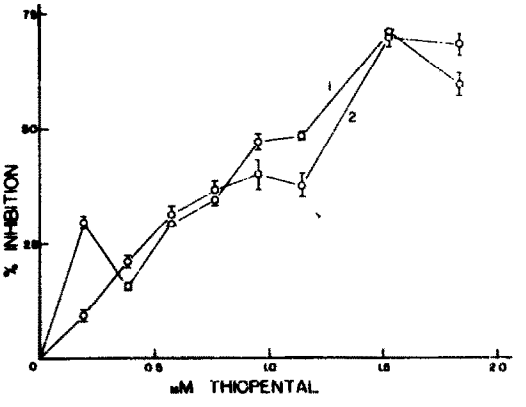

Fig. 4. Dose-related inhibition of GABA-T reaction by thiopental. Line 1 represents data where GABA is varied and line 2 represents data where 2-OG is varied.

Thiopental caused a similar inhibition of GABA-T activity (Fig. 4) with limiting concentrations of GABA or 2-OG. The maximal inhibition appeared to be near 70 per cent. Thiopental, at concentrations higher than 2 mM, caused precipitation in the reaction mixture and data for higher concentrations could not be obtained.

DISCUSSION

Although the time course of the rat brain synaptosomal GABA-T reaction in this study was not exactly linear (Fig. 1), it was consistent and reproducible. After an initial lag period, which is characteristic of product determination in a coupled enzyme assay, the rate increased gradually with time and maximal rates were not reached until after 1 hr of preincubation. The initial dip in the absorbancy at 340 nm could be reduced, but not eliminated, by adding trace amounts of NADH during preincubation. For practical reasons, reaction rate calculations were based on the absorbancy difference at 340 nm between 10 and 40 min of incubation. Although the reaction rate was not quite linear in this time period, the results in Table 1 show that rates thus calculated are proportional to the amounts of synaptosomes added. Since the other reaction product, glutamate, could be utilized to reduce NAD, glutamate was substituted for GABA to test for glutamate dehydrogenase activity. There was no measurable reaction with glutamate, suggesting that no significant GDH activity was present to complicate the reaction.

In this study, the apparent K_m value of GABA-T for GABA (16.0 mM) was found within the K_m values reported previously: 5.3 and 6.5 mM, respectively, from rat brain "synaptosomal" and "cytoplasmic-mitochondrial" enzymes [12], 12 mM from mouse brain [13], 4.55 mM from rat brain [14], 4 mM from rat brain [15], 3 mM from mouse brain [16], 2.6 and 1.3 mM, respectively, from mouse brain "synaptosomal" and "cytoplasmic-mitochondrial" preparations [17], 1.1 mM from mouse brain [18], 1.25 and 0.8 mM, respectively, from rat brain "free mitochondria" and "synaptosomal mitochondria" [19], and 0.2 mM from rat brain [20]. The apparent K_m for 2-OG (0.35 mM) from this study was consistent with the lower values reported previously: 5.5 mM [15], 4.9 mM [14], 4 mM [16], 2 mM [13], 0.25 mM [18], 0.5 mM ("free mitochondria") and 0.25 mM ("synaptosomal mitochondria") [19], and 0.2 mM [20]. Previous reports on the inhibitory action of high concentrations of 2-OG [15, 19] were confirmed.

The maximal inhibition of GABA-T activity by thiopental was around $\frac{2}{3}$ of its total activity (Fig. 4) and the mechanism was noncompetitive. Either $\frac{2}{3}$ of all the enzyme molecules or $\frac{2}{3}$ of the activity of each molecule was inhibited.

Since two types of GABA-T from two mitochondrial populations were demonstrated recently [12, 17, 19] and our synaptosomal preparation was not free of small mitochondria (about 5 per cent contamination by vehicle control), it is possible that this maximum inhibition reflected differential inhibition by thiopental on GABA-T in these two types of mitochondria. Only further experimentation can clarify this problem.

An inhibitory action of barbiturates on GABA-T has not been reported previously. Pentobarbital has been reported not to inhibit GABA-T [2, 3]. We [6] found that 10 per cent inhibition of GABA-T from solubilized rat brain synaptosomes occurred at 0.17 mM thiopental, 0.75 mM pentobarbital and 0.80 mM phenobarbital.

The K_i for thiopental found in this study was 1.01 or 1.15 mM, respectively, when either GABA or 2-OG was the limiting substrate. These values are not different statistically and can be averaged to 1.1 mM. The pharmacological relevance of this value is difficult to assess since there are no data available as to the *in vivo* anesthetic concentration of thiopental in the rat. An indirect estimate, albeit dubious, can be made. The average thiopental concentration in four brain areas in the dog 20 min after an i.v. injection of 25 mg pentothal/kg body weight was reported to be 28.2 mg thiopental/kg of brain [23]. Taking into

account the molecular weight of thiopental (mol. wt. = 242) and brain water content of 77 per cent [22], a thiopental concentration of 0.15 mM in brain could be calculated. The percentage of thiopental remaining in human brain (CNS) 20 min after a single i.v. dose was estimated to be 19 per cent of the maximal thiopental concentration in brain [23]. Combining 0.15 mM with 19 per cent of maximal concentration yields an anesthetic thiopental concentration of about 0.8 mM. This value is less than the brain concentration of thiopental of 3 mM 1 min after an i.v. injection of 25 mg/kg of thiopental [24]. Both these values approximate the K_i value of 1.1. mM reported in this study.

These results demonstrate that GABA-T activity can be inhibited by concentrations of thiopental attained clinically. This inhibition may lead to an increase in GABA content in synaptic areas without an increase in total brain GABA content. Some workers estimate the synaptic pool of GABA to be 10 per cent [25] to 30 per cent [26], and significant increases in this pool may occur but be hidden by the much larger non-synaptic pool. It is known that some agents increase brain GABA content several-fold without causing anesthesia [27–32]. They may act on GABA pools in non-synaptic sites. The important factor is the GABA concentration at the synapse. Neither the means to estimate that quantity nor the techniques to determine it are available at this time.

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