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# EFFECT OF 6-AZAURACIL, AND OF CERTAIN STRUCTURALLY SIMILAR COMPOUNDS, ON THREE PYRIDOXAL-PHOSPHATE REQUIRING ENZYMES INVOLVED IN NEUROTRANSMITTER METABOLISM*

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**Abstract**—6-Azauracil, which is reported to have an hypnotic effect in man, rat and mouse, is a non-competitive inhibitor of gamma-aminobutyric acid (GABA)-transaminase activity in rodent and human brain extracts. The $K_i$ for the rat brain activity is $5 \times 10^{-4}$ M. GABA-transaminase is also non-competitively inhibited by a number of other compounds—both drugs and natural substances—which are reported to affect arousal and which, like 6-azauracil, include within their structure a pyrimidine or pyrimidine-like ring. None of these compounds, however, is as potent an inhibitor of the transaminase as 6-azauracil. Glutamic acid decarboxylase is inhibited only weakly (and non-competitively) by 6-azauracil and, to an even lesser extent, by the other substances tested. None of the compounds under study caused appreciable inhibition of DOPA decarboxylase except under conditions where the enzyme proved highly unstable, and even under these conditions the inhibition was weak.

The pyrimidine analogue 6-azauracil and its riboside (6-AZUR) have been employed, both experimentally and clinically, as antineoplastic agents [1–5; *inter alia*], and the 2′,3′,5′-triacetyl derivative of the riboside (azaribine) has been tested extensively as an antipsoriatic [6, 7; *inter alia*]. The ribotide of 6-azauracil, 6-azauridine-5′-monophosphate (6-AzaUMP), is a potent competitive inhibitor of the final enzyme (orotidine-5′-monophosphate decarboxylase) in the synthetic sequence leading to uridine-5′-monophosphate (UMP), [1, 8; *inter alia*]. This property of the ribotide is believed to form the basis for the antineoplastic and antipsoriatic activities of 6-azauracil and its derivatives [1, 4, 6, 7].

One of the factors which has restricted the use of these drugs is reversible neurotoxicity characterized mainly (but not exclusively) by somnolence, hypersomnia, and, at high doses, in animals (and occasionally in man) by coma [1, 2, 9–11‡]. Published data render it virtually certain that these symptoms follow from some property of 6-azauracil which is unrelated to the inhibition of UMP synthesis produced by 6-AzaUMP [1–3, 12–15].

Patients [16] and animals [17] receiving azaribine develop elevated blood levels of several amino acids which are each catabolized by a different enzyme sequence. In every case, however, at least one pyridoxal-phosphate (PLP) requiring enzyme occurs within the sequence. The elevated amino acids include both essential and non-essential ones, and this latter group includes two non-structural amino acids (homocystine and beta-alanine). On the basis of these findings, it has been suggested [16, 17] that "azaribine or one of its metabolites may be acting as a pyridoxal phosphate inhibitor" and that a functional deficiency of PLP may account for the "sedation (and) psychic retardation."

Since PLP is known to serve as a cofactor for several enzymes which play a role in neurotransmitter metabolism, we have examined the effect of 6-azauracil (and several structurally similar compounds) on three such enzymes: glutamic acid decarboxylase (GAD) which effects the synthesis of gamma-aminobutyric acid (GABA); GABA-transaminase (GABA-T) which is the main enzyme of GABA catabolism and which catalyzes the replacement of the keto group on the alpha-carbon of alpha-ketoglutarate with the gamma-amino group of GABA (thus forming glutamic acid and succinic acid semialdehyde); and L-dihydroxyphenylalanine decarboxylase (DOPAdcase) which effects the synthesis of dopamine.

## MATERIALS AND METHODS

Two convulsant barbiturates [18, 19], CHEB [5-(2-cyclohexylideneëthyl)-5-ethyl barbituric acid] and DMBB [5-(1,3-dimethylbutyl)-5-ethyl barbituric acid], were gifts from Dr. Hall Downes, University of Oregon Medical School, Portland, OR. All other chemicals and reagents were obtained commercially and were of the highest grade available. Adult Wistar rats were purchased from Camm Research, Wayne, NJ, and adult male C57/BL6J mice were obtained from the Jackson Laboratories, Bar Harbor, ME. Brains from adult human subjects, free from known central nervous system diseases, were obtained post-mortem, and were

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‡ An untitled compilation (prepared by the staff of Parke, Davis & Co.) of clinical experience with azaribine at a number of North American clinics. Dr. Fischetti of Parke Davis & Co. kindly made these analyses available to me, and assisted me in interpreting the results.

provided by the Neuropathology Service of the Columbia-Presbyterian Medical Center. Brain extracts were prepared by the method of Beart *et al.* [20] for all assays; however, for the assays for GAD and DOPAdcase the high speed centrifugation step was omitted, and the extracting medium was 0.26 M sodium phosphate buffer at pH 6.8 and 7.0 respectively. In experiments where the extract was subjected to gel filtration, a 2.5 × 25 cm column of Sephadex G-25 was used. All three enzymes were assayed radiochemically, employing $^{14}C$-substrates. GABA-T was assayed by either of two methods. Assay I [20] measures the formation of labeled succinate semialdehyde (after its enzymatic conversion to labeled succinic acid) from labeled GABA. Assay II [21] measures the formation, in the presence of unlabeled GABA, of labeled glutamate from labeled alpha-ketoglutarate. We have modified the protocol for this latter assay slightly, so that we can express enzyme activity in terms of the GABA-dependent generation of labeled alpha-ketoglutarate. GAD activity was also determined by either of two methods. Assay I [22] measures the liberation of $^{14}CO_2$ from [1-$^{14}C$]glutamic acid, and Assay II, which is similar to an assay reported by others [23], measures the formation of labeled GABA from uniformly labeled glutamate. In our version of this latter assay, the labeled glutamate was separated by paper chromatography from the labeled product (GABA). The chromatography was carried out for 18 hr on Whatman No. 3 MM paper, employing as a solvent *t*-butylalcohol–methylethylketone–water–ammonium hydroxide (14.8 M) (40:30:20:10). The respective $R_f$ values for glutamate and GABA were 0.14 and 0.38. The levels of enzyme activity obtained with this assay were identical in the presence and absence of 1 mM unlabeled GABA as a cold trap. Assays I and II yielded identical estimates of specific GAD activity, and, in both cases, 95–99 per cent of the activity was inhibited by 1 mM aminooxyacetic acid, a PLP antagonist which is known to inhibit GAD [24]. In the case of both GABA-T and GAD, the assay denoted as Assay I was employed except where otherwise specified. The assay for DOPAdcase [25] measures the release of $^{14}CO_2$ from [1-$^{14}C$]-DL-DOPA.

## RESULTS

*GAD.* Table 1 reveals that only 6-azauracil, among the compounds tested, caused appreciable inhibition of GAD activity. Results essentially identical to those in Table 1 have also been obtained with extracts of mouse brain and human brain. (In the case of human brain, however, cytosine, barbital and the two convulsant barbiturates have not been tested thus far.) The inhibitory effects on GAD activity of four of the compounds listed in Table 1 (uracil, 6-azauracil, barbital and phenobarbital) were examined by Assay II as well as by Assay I. The two assays gave very similar results. For example, using aliquots from the same brain extract, 6-azauracil caused 24.3 ± 0.3 per cent inhibition by Assay I and 29.2 ± 5.2 per cent by Assay II.

The percentage inhibition of GAD by 6-azauracil (or by the other agents shown in Table 1) was not increased by diminishing the concentration of exogenous PLP, added to the reaction mixture, from 0.236 mM to zero. On the other hand, such a reduction in the final concentration of PLP causes about a 2-fold fall in the quantity of glutamic acid decarboxylated during the 30 min incubation period. Hence, it is unlikely that the inhibition of GAD by 6-azauracil is competitive with PLP. Similarly, reducing the final concentration of added glutamic acid from 3.1 mM to 0.31 mM did not lead to an increase in the percentage of inhibition of GAD

Table 1. Effect of 6-azauracil, and a number of compounds which have a structural similarity to 6-azauracil, on three enzyme activities of rat brain *

| Additive to reaction mixture | GAD† Specific activity (nmoles/min/mg) | GAD† % Inhibition ascribable to presence of additive | GABA-T‡ Specific activity (nmoles/min/mg) | GABA-T‡ % Inhibition ascribable to presence of additive | DOPA decarboxylase§ Specific activity (nmoles/hr/mg) | DOPA decarboxylase§ % Inhibition ascribable to presence of additive |
|---|---|---|---|---|---|---|
| None | 2.05 (± 0.02) | 0 | 2.17(± 0.09) | 0 | 12.2 (± 0.20) | 0 |
| Uracil | 2.09 (± 0.02) | −2 | 1.53 (± 0.95) | 29.4 | 12.3 (± 0.09) | 0 |
| 6-Azauracil | 1.33 (± 0.01) | 35 | 0.18 (± 0) | 91.7 | 12.3 (± 0.39) | 0 |
| Thymine | 2.01 (± 0.02) | 2 | 1.47 (± 0.02) | 32.1 | 11.9 (± 0.57) | 2.1 |
| Cytosine | 1.99 (± 0.04) | 5 | 2.01 (± 0.02) | 7.4 | | |
| Barbital | 1.97 (± 0.01) | 4 | 2.04 (±0.05) | 6.0 | | |
| Phenobarbital | 2.03 (± 0.05) | 1 | 1.65 (± 0.04) | 23.8 | 10.1 (± 0.05) | 17.9 |
| CHEB‖ | 1.99 (±0.04) | 3 | 1.93 (± 0.06) | 10.8 | | |
| DMBB‖ | 2.00 (± 0.04) | 2 | 1.72 (± 0.05) | 20.8 | | |

* GAD denotes glutamate decarboxylase, and GABA-T denotes GABA-transaminase. The numbers in parentheses represent the standard error of the mean which precedes them. Each standard error is based on the mean square between triplicate reaction tubes. (±0) denotes a standard error of less than 0.01.

† GAD was measured by Assay I as described under Materials and Methods. In each case the concentration of the additive was 5 mM, and each of the reaction mixtures contained 2.29 mg of brain protein.

‡ GABA-T was measured by Assay I as described under Materials and Methods. In every case, the concentration of the additive was 6.60 mM, and each of the reaction mixtures contained 0.45 mg of brain protein.

§ DOPA decarboxylase was assayed as described under Materials and Methods, except that in this particular experiment the enzyme was extracted, prior to assay, as an acetone powder. (Results similar to these were also obtained with crude homogenates.) In each case the concentration of the additive was 5.25 mM, and each of the reaction mixtures contained 2.40 mg of brain protein.

‖ CHEB (Downes and Franz [18]) and DMBB (Daves *et al.* [19]) are convulsant barbiturates.

activity caused by 6-azauracil (or by the other agents indicated in Table 1). However, such a reduction in substrate concentration causes about a 5- to 6-fold fall in the quantity of glutamic acid decarboxylated during the 30-min incubation period. Therefore, it is unlikely that the inhibition of GAD by 6-azauracil is competitive with glutamic acid either. In the case of 6-azauracil, the experiments on the effect of reduced substrate or cofactor concentration were performed by employing both Assay I and Assay II, and the two assays yielded virtually identical results.

*GABA-T.* Table 1 reveals that all the heterocyclic compounds tested caused measurable inhibition of GABA-T. The levels of inhibition shown in Table 1 have proven repeatable even in the case of compounds causing only slight inhibition. The most potent inhibitor, among the compounds tested, was clearly 6-azauracil, while the inhibition due to cytosine, barbital and CHEB was negligible. As with GAD, data nearly identical to those shown in Table 1 have been obtained with extracts of mouse brain and of human brain, except that in the case of human brain cytosine, barbital and the two convulsant barbiturates were not tested. In the case of rat brain, the inhibition by 6-azauracil of GABA-T activity, measured by Assay I, was confirmed with Assay II. Employing this latter assay, the GABA-dependent generation of labeled glutamic acid proved to be inhibited by 6.6 mM 6-azauracil, and to about the same extent (96.7 ± 0.15 per cent) as the GABA-T activity measured by Assay I (Table 1).

A 10-fold reduction in the concentration of GABA (from 0.67 mM to 0.067 mM) caused a 5-fold reduction in the quantity of GABA deaminated during the 30-min incubation period, but had no effect on the percentage of inhibition caused by the various compounds shown in Table 1. Figure 1 shows that, in the case of 6-azauracil, the inhibition is indeed non-competitive [25] with respect to GABA. From the data in Fig. 1, and from similar experiments, we have calculated [26] that the $K_i$ of the transaminase for 6-azauracil is $5 \times 10^{-4}$ M. The corresponding $K_i$ for the other agents listed in Table 1 was about ten times this value, except in the case of cytosine, barbital and CHEB where the level of inhibition was too low to permit an estimate of the $K_i$.

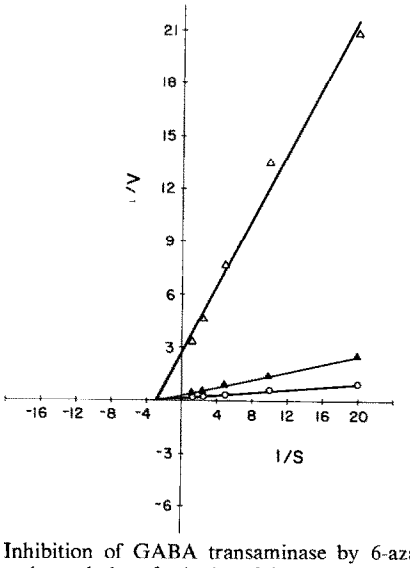


Fig. 1. Inhibition of GABA transaminase by 6-azauracil. Double-reciprocal plot of velocity of GABA transamination, catalyzed by a rat brain extract, vs GABA concentration in the reaction mixture: effect of 6-azauracil. GABA-T was measured by Assay I as described under Materials and Methods except that, immediately prior to assay, the mitochondrial fraction (of the extract) was subjected to Sephadex gel filtration (cf. Materials and Methods). The ordinate measures the reciprocal of reaction velocity whose units are nmoles GABA deaminated/mg of brain protein/min. The abscissa measures the reciprocal of GABA concentration expressed as mmoles/liter. Every reaction flask contained rat brain extract corresponding to 0.069 mg of protein. Key: (○——○) no 6-azauracil present; (▲——▲) 0.66 mM 6-azauracil; and (△——△) 6.6 mM 6-azauracil.

The effect of a 40-fold reduction (from 0.44 mM to 0.011 mM) in the concentration of the other co-substrate (alpha-ketoglutarate) caused a 4.5-fold reduction in the quantity of GABA deaminated during the 30-min incubation period. However, lowering the alpha-ketoglutarate concentration failed to have a measurable effect on the percentage of inhibition produced by uracil, 6-azauracil, thymine or phenobarbital. (The remaining compounds listed in Table 1 were not studied in this respect.) Hence, at least with respect to these four substances, the inhibition observed is unlikely to be competitive with alpha-ketoglutarate.

Omitting added PLP from the reaction mixture, or increasing the concentration of added PLP from 0.06 mM to 3 mM, also did not affect the percentage of inhibition caused by the compounds listed in Table 1. This result was obtained even when the extract was dialyzed for 48 hr or was subjected to Sephadex gel filtration prior to incubation with substrate. In most experiments (including those which involved prior dialysis or gel filtration of the enzyme), the omission of PLP from the reaction mixture did not in itself affect the absolute level of enzyme activity which was measured. Presumably, the endogenous PLP within the extract is tightly bound to the enzyme. Highly purified GABA-T from mouse brain is in fact known to employ PLP as a cofactor, and has a $K_m$ for this substance of $5 \times 10^{-8}$ M [24]. In any case, our procedures—Sephadex gel filtration and dialysis—are likely to have effected a marked reduction in the endogenous unbound PLP concentration of our extracts. The fact that, even following gel filtration or dialysis of the extract, the omission of added PLP failed to influence the percentage of inhibition of GABA-T by any of the agents listed in Table 1 suggests that the inhibition is not competitive with PLP either.

GABA-T, however, is a mitochondrial enzyme. Hence, it is conceivable that 6-azauracil might be able to permeate the mitochondria and displace, at a catalytic site, endogenous PLP (or one or both cosubstrates) trapped by the mitochondrial membrane. On the other hand, added PLP or substrate, which is in solution external to the mitochondria, might not enter the mitochondria as readily as 6-azauracil. As a result, the seemingly non-competitive nature of the inhibition might be spurious. This possible fallacy applies particularly to PLP, since we were unable to demonstrate dependence of the reaction upon an exogenously supplied source of PLP. In any case, to explore the matter further, we employed 1% triton in an effort to release GABA-T from the mitochondria. We first spun the rat brain homogenate at 17,000 *g* for 10 min. The resulting

pellet was then resuspended in buffer, and the suspension was divided equally among several tubes. Triton was added to some of the tubes. The suspensions were then spun again at 17,000 *g* for 10 min. In the tubes containing 1% triton, the ratio of activity in the supernatant fracture to that in the pellet was 16: 1, whereas in the absence of triton, the ratio was 1:7. Hence, it appeared that triton released GABA-T from the mitochondria. We then subjected the 17,000 *g* 10 min supernatant fluid from the tubes containing triton to Sephadex gel filtration. Under these circumstances, in the absence of added PLP, GABA-T had only about half the activity measured in the presence of 3 mM added PLP. However, the presence or absence of added PLP did not affect the percentage of inhibition caused by 6-azauracil. In the absence of added PLP, 0.67 mM and 6.7 mM 6-azauracil caused 51.1 and 91.7 per cent inhibition of GABA-T activity respectively. In the presence of 3 mM added PLP, the corresponding figures were 49.9 and 91.9 per cent. Similarly, reducing the concentration (0.44 mM) of alpha-ketoglutarate by 10-fold led to a 7-fold fall in GABA-T activity, but had no effect on the percentage inhibition due to 6-azauracil. Finally, reducing the GABA concentration from 0.67 mM to 0.067 mM led to a 4-fold reduction in enzyme activity, but, again, did not affect the percentage inhibition caused by 6-azauracil. We conclude, therefore, that the inhibition of GABA-T by 6-azauracil is not likely to be competitive with the two substrates or the cofactor, even in a preparation where the enzyme can reasonably be presumed to have been released from mitochondria.

Employing Assay I for GABA-T, the radioactivity in the product was found to be directly proportional to the duration of the incubation, both in the presence and absence of 6-azauracil, for up to 20 min. After 20 min, however, enzyme activity per unit time fell progressively. Kinetic plots revealed that 6-azauracil, and to a somewhat lesser extent thymine, decelerated the decay of enzyme activity, a phenomenon which we presume reflects the stabilization of the enzyme by the inhibitory compounds. Finally, we wish to emphasize that GABA-T activity was directly proportional to protein concentration both in the presence and absence of 6-azauracil (Fig. 2). This result (together with our other studies) supports the view that the inhibition of GABA-T by 6-azauracil is reversible (cf. Ref. 26).

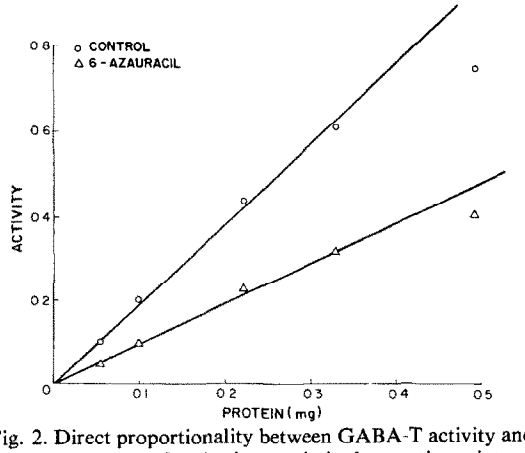


Fig. 2. Direct proportionality between GABA-T activity and the concentration of rat brain protein in the reaction mixture. GABA-T activity was measured both in the presence and absence of 0.67 mM 6-azauracil, and is expressed as nmoles GABA deaminated/mg of brain protein/min.

*DOPAdcase.* Table 1 summarizes the effects of uracil, 6-azauracil, thymine and phenobarbital on DOPAdcase. As can be seen, in the presence of PLP, only phenobarbital, among the compounds tested, caused appreciable inhibition of enzyme activity; the inhibition due to phenobarbital, however, was slight (and became undetectable when the phenobarbital concentration was reduced 10-fold to $5.3 \times 10^{-4}$ M). In the absence of added PLP, phenobarbital caused significantly more inhibition (66 per cent), and there was appreciable (and repeatable) inhibition due to 6-azauracil and thymine (18.6 and 20.6 per cent, respectively), although even under these conditions uracil remained inactive. However, when PLP was omitted from the reaction mixture, the enzyme activity proved extremely unstable. The relatively low level of inhibition due to each of these compounds, even in the absence of added PLP, together with the apparent influence of PLP on the stability of the enzyme activity, rendered it impossible to determine whether the inhibition was indeed competitive with PLP.

Reducing the concentration of DOPA in the reaction mixture from 4 mM to 0.4 mM caused a 40 per cent fall in the quantity of DOPA decarboxylated during the 30-min incubation period, but had no effect on the percentage of inhibition of enzyme activity caused by the agents listed in Table 1. (These latter experiments were performed, of course, in the presence of added PLP.)

In any case, it is clear that DOPA decarboxylase is inhibited to a much lesser extent than GABA-T by 6-azauracil, and, at least in the presence of PLP, the same would appear to be true of the other compounds listed in Table 1.

## DISCUSSION

Because of the observations of Slavik *et al.* [16], which we summarized earlier, we suspected initially that 6-azauracil might: (1) strongly inhibit all PLP-requiring enzymes; and (2) compete with PLP for a combining site on each of these enzymes. However, our results appear to indicate that both of these suspicions were wrong. Of the three enzymes we studied, only GABA-T was inhibited strongly by 6-azauracil, and, at least in the case of GAD and GABA-T, the inhibition did not seem to involve competition between 6-azauracil and PLP. Nonetheless, on the basis of the observations of Slavik *et al.* [16], we presume that a relationship exists between the inhibition of GABA-T by 6-azauracil and the requirement of the enzyme for PLP as a cofactor. However, the nature of the relationship, if it in fact exists, is not apparent.

We have designated provisionally the combining site on the transaminase for 6-azauracil as the "heterocyclic ring receptor site (HRRS)." By such a nomenclature we intend to imply only that certain heterocyclic rings interact with the site, and that this interactive site on the enzyme appears to be separate from the sites that combine with the reactants. We presume that the weaker inhibition of the transaminase by uracil, thymine and certain of the barbiturates we have tested results from an interaction at the same site as the one where 6-azauracil interacts. We base our presumption on the fact that these molecules resemble 6-azauracil in

structure and, like 6-azauracil, cause an inhibition of the transaminase which appears to be non-competitive with cofactor or substrate.* However, experiments which might bear more directly on the identity of the interactive site for each compound have not been performed yet.

The non-competitive inhibition of GABA-T by 6-azauracil and the other heterocyclic compounds tested implies that either these compounds prevent GABA from occupying the GABA-combining site on the enzyme, or else they prevent the conformational change which the enzyme would normally undergo in response to a collision with GABA (in the presence of the other reactants) (cf. Ref. 26). Like GABA-T the GABA receptor protein [27] and the GABA uptake protein [27] specifically recognize GABA, and presumably undergo a conformational change in response to a collision with GABA. It is perhaps unlikely that each of the molecules sharing these properties arose by a completely independent evolutionary route. If these molecules have some kind of evolutionary relationship, it might follow that the GABA receptor or uptake protein includes a site, homologous to the HRRS on GABA-T, which interacts with heterocyclic rings. There is, of course, an extensive literature implying that GABA may serve as a depressant of brain excitability in mammals [28–33; *inter alia*], and several authors have suggested, or published evidence suggesting, that GABA is somehow involved in mediating the hypnotic effect of the barbiturates [34–36; *inter alia*].

Present evidence [24, 37, 38] suggests that GABA-T is likely to be identical with the enzyme which deaminates beta-alanine. It is of interest that an infant has been described [28, 29] who was affected with striking somnolence and episodic seizures, and who had elevated levels of both GABA and beta-alanine in blood, brain and kidney. Indirect but persuasive evidence indicated that the infant was likely to be deficient in the ability to deaminate these two amino acids [28, 29]. One possible pathogenesis for the somnolence of the patient is his elevated brain levels of GABA (and/or of beta-alanine) [28, 29]. Our observation that 6-azauracil, a compound with known hypnotic activity in man and mouse, inhibits GABA-T non-competitively seems to us to lend some support to that interpretation. In this connection, it is worth noting that our estimate of the $K_i$ of GABA-T for 6-azauracil is only about one-tenth of the parenteral dose, per g body water, necessary to cause a 50 per cent reduction in the spontaneous activity of the mouse [14].

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* In the case of barbital, the two convulsant barbiturates (CHEB and DMBB) and cytosine, we have studied thus far only the effect of lowering the concentration of GABA and of PLP on the level of inhibition. We have not examined yet the effect of reducing the alpha-ketoglutarate concentration. In this connection, it will be recalled that lowering the concentration of alpha-ketoglutarate did not influence the percentage inhibition of GABA-T by 6-azauracil, uracil, thymine or phenobarbital.

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