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COMMENTARY

INHIBITORS OF GABA METABOLISM

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The recognition of γ-aminobutyric acid (GABA) as a major inhibitory neurotransmitter in the mammalian central nervous system [1] has stimulated the search for drugs such as GABA receptor agonists, GABA uptake inhibitors and inactivators of 4-aminobutyrate:2-oxoglutarate aminotransferase (GABA-T, EC 2.6.1.19), the GABA catabolizing enzyme, which may potentiate GABA neurotransmission. Such agents may be useful in treating several diseases in which a deficiency of GABA function has been demonstrated or implicated, e.g. Huntington's disease [2–4], tardive dyskinesia [5], Parkinsonism [6], epilepsy and schizophrenia [7]. Of course, as GABA itself does not readily cross the blood brain barrier, its oral administration would not correct a deficiency of GABA in brain [8]. During the last ten years a new concept of enzyme inhibition has been enunciated. This concept requires that the inhibitor contain a latent reactive functionality which is liberated as a result of the target enzyme's own mechanism of action. Such inhibitors, which have been designated "K_{cat}" inhibitors [9] or "suicide enzyme inactivators" [10], are expected to be highly-specific because they should inhibit only those enzymes which can accept them as substrates. Several inhibitors, which function by a mechanism requiring activation by GABA-T prior to that enzyme's irreversible inactivation, have recently been designed and synthesized [11–13] or isolated from natural sources [14]. It is the intent of this Commentary to describe the status of inhibitors of GABA metabolism from a mechanistic, and hence specificity, viewpoint. Another Commentary entitled "GABA agonists and antagonists" will appear in a forthcoming issue of this journal [15].

GABA-T, being a pyridoxal phosphate (PyCHO)-dependent enzyme, operates via a tautomerism of the Schiff base formed between GABA and PyCHO. The Schiff base function activates the adjacent C–H bond, so that proton abstraction is facilitated. Reprotonation leads to the tautomeric Schiff base which is hydrolyzed to the products, succinic semialdehyde and pyridoxamine (Fig. 1). In order for further transamination of GABA to proceed, the holoenzyme must be converted back to the pyridoxal form. This is achieved with the advent of another substrate, α-ketoglutarate, which undergoes Schiff base formation with the pyridoxamine. The sequence shown in Fig. 1 is then reversed with the amino group of pyridoxamine being transferred to α-ketoglutarate, thus regenerating PyCHO and glutamic acid [16].

In view of the dependency of this mechanism on Schiff base formation, it is not surprising that the first reported inhibitors of GABA-T were compounds chosen to form a more stable Schiff base with PyCHO, than could GABA itself. By this reasoning, the non-specific aldehyde antagonists hydroxylamine (NH_2OH) [17], hydrazine (NH_2NH_2) [18] and an alkyl-substituted hydroxylamine, aminooxyacetic acid ($HOOCCH_2ONH_2$, AOAA) [19], which more closely resembles GABA itself in chain length and charge separation, were introduced. Despite their inherent lack of discrimination

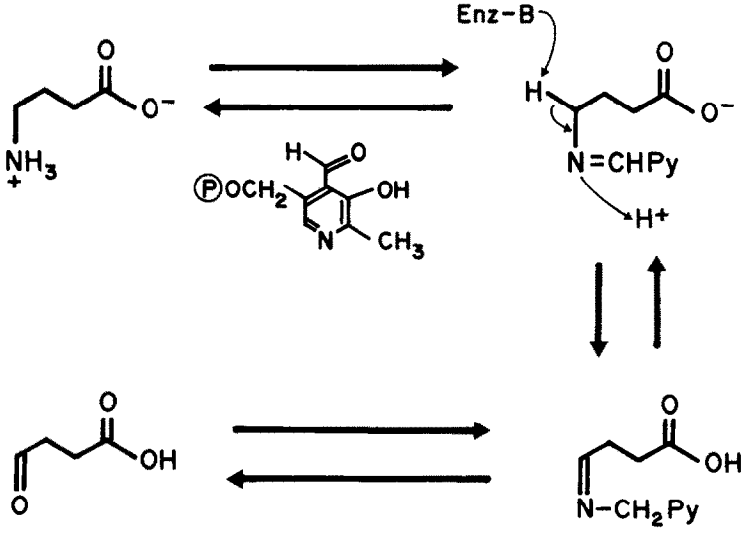


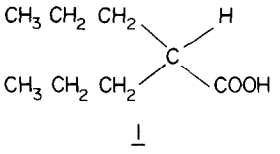
Fig. 1.

among PyCHO-dependent enzymes, administration of each of these compounds *in vivo* results in an increase in brain GABA concentrations; the elevation of GABA levels induced by AOAA being the greatest and more sustained.

Glutamic acid decarboxylase, (GAD, EC 4.1.1.15), the GABA biosynthesizing enzyme, is also PyCHO-dependent, and is subject to inhibition by AOAA, *in vitro* and *in vivo* [20, 21]. That AOAA is still able to induce an increase in brain GABA content, while inhibiting GAD, reflects a lower degree of inhibition of this enzyme, compared to that of GABA-T, *in vivo*.

AOAA, as well as hydrazine, exerts an anticonvulsant action at low doses, while at higher doses it has been found to be excitatory [22]. Since at higher doses of AOAA, GAD activity is considerably reduced, the conclusion has been drawn that the susceptibility to induced convulsions in animals depends not only on GABA cencentrations, which may be highly elevated, but also on GAD activity, which determines the rate at which GABA is introduced into the synaptic cleft. Such a correlation of occurrence of convulsions with a decrease in GAD activity has been observed with a variety of agents which inhibit both GABA-T and GAD, as a result of their property of being general PyCHO antagonists. Thus AOAA [22], hydrazine [23], glutamic acid γ-hydrazide [24] and isonicotinic acid hydrazide [25] have all been shown to be convulsant agents at doses where brain GAD activity is reduced, while GABA concentrations are elevated. Indeed, an empirical mathematical expression has been devised to reflect the degree of brain excitability as a function of brain GABA concentration and GAD activity, the latter term being the dominant expression [23]. Since these drugs have anticonvulsant properties at low doses, where GAD activity is not seriously affected, while brain GABA concentrations are elevated as a result of GABA-T inhibition, the search for more specific GABA-T inhibitors as potential anticonvulsant agents has continued.

In 1968 Van Gelder introduced hydrazinopropionic acid ($HOOCCH_2CH_2NHNH_2$), a substituted hydrazine which retains such characteristics of the GABA molecule as conformational flexibility and zwitterionic charge distribution [26]. In accordance with this molecular similarity to GABA plus the possibility of essentially irreversible hydrazone formation with PyCHO at the active site, hydrazinopropionic acid has been found *in vitro* to be potent inhibitor of both mammalian GABA-T and GAD, the inhibition kinetics being compatible with those of a competitive inhibitor. The inhibition of GABA-T is not reversed by PyCHO suggesting an extremely high affinity of the pyridoxal-hydrazone for the enzyme. *In vivo* GAD is inhibited to a lesser extent than is GABA-T, so that brain GABA levels rise dramatically upon administration of the drug and remain elevated for at least 16 hours. Although a systematic study of the anticonvulsant and convulsant properties of hydrazinopropionic acid has not been reported, it appears that, like AOAA, it produces sedation at low doses (1–20 mg/kg subcutaneous), while higher doses (30 mg/kg) result in convulsions and death [26]. Although one of the most potent GABA-T inhibitors known ($K_I = 2.35 \times 10^{-7}$ M, while K_m for GABA $= 2.65 \times 10^{-2}$ M), the drug has not been widely used in studying the effects of inhibition of GABA catabolism, as has AOAA, presumably owing to its general lack of availability and stability.



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Di-n-propyl acetate (DPA, Structure 1) is an effective anticonvulsant in several animal seizure models [27], and in the treatment of petit mal epilepsy [28]. The mechanism of its anticonvulsant action has been suggested to result from a competitive inhibition of GABA-T, with the consequent elevation of brain GABA concentrations. Its inhibitory effect on GABA-T is rather weak ($K_I = 1.4 \times 10^{-3}$), and with an intramuscular dose of 400 mg/kg in mice, whole brain GABA levels are maximally increased by 37 per cent [29]. This is to be compared to a 400 per cent increase on administration of an anticonvulsant dose of AOAA (25 mg/kg, i.p.). It is therefore conjectural that GABA-T inhibition is the mechanism responsible for the anticonvulsant effects of DPA. It has been noted that administration of DPA decreases the brain concentration of the putative excitatory neurotransmitter, aspartate, in mice [30] and in rats [31], to a similar degree to which it raises brain GABA concentrations. The concentrations of DPA found effective clinically, however, are only a fraction of those found necessary in animals to perturb brain aspartate or GABA levels, and it would seem that the mechanism of the anticonvulsant effect of DPA and of other branched-chain fatty acids [32] remains to be elucidated.

Cycloserine (Structure 2) has been shown to be an irreversible inhibitor of a number of PyCHO-dependent enzymes, including GABA-T and GAD [33, 34], and again, as its effect on GABA-T is more pronounced than on GAD, it is able to induce an increase in whole brain GABA concentrations *in vivo* [33]. However, as it inhibits other PyCHO-dependent enzymes, such as aspartate aminotransferase and alanine aminotransferase [35], its usefulness in the study of the functional role of GABA in the CNS has been precluded. An examination of the structure of cycloserine (2) suggests that its action towards PyCHO-dependent enzymes would not be of the hydrazine or hydroxylamine type. Indeed, it has been suggested that cycloserine (2) is a substrate for these enzymes, and that irreversible inhibition results from the formation of an alkylating species generated by the enzyme's own mechanism of action, as shown in Fig. 2 [34].

Thus, if cycloserine (2) is accepted as a substrate by GABA-T, Schiff base formation with PyCHO should occur facilitating cleavage of the C-H bond α to the Schiff base (compare with Fig. 1). Reprotonation would then lead to the acylating species 3, which could react with an appropriately-situated nucleophilic group (Nu) in the active site, leading to covalent bond formation and hence irreversible inactivation. Cycloserine (2) would thus be the first example of an irreversible inhibitor of GABA-T which requires transformation by that enzyme prior to its irreversible inhibition. The enzyme hence commits suicide by its own mode of action.

The substituted cycloserine 4, which is a GABA analog, has also been shown to be an irreversible

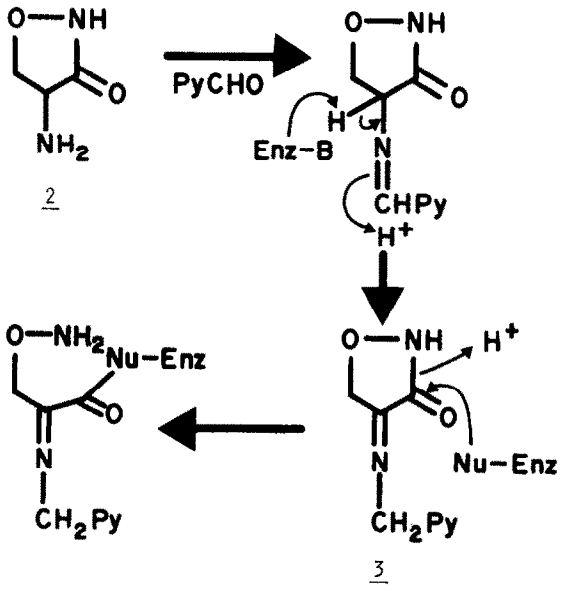

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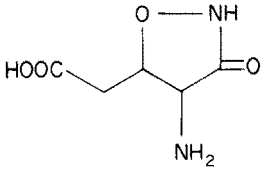
Fig. 2.



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inhibitor of GABA-T, the inactivation process being time-dependent [35]. Data as to its *in vivo* effects or specificity have not yet been published.

Ethanolamine-*O*-sulfate (EOS, 5), another enzyme-activated irreversible inhibitor of GABA-T, was designed by Fowler and John [11] to take advantage of the proton abstraction step involved in the transamination of GABA by GABA-T (Fig. 1). Thus EOS (5), a substrate analog, undergoes Schiff base formation at the active site of GABA-T. The subsequent cleavage of the α C-H bond than induces β-elimination of sulfate to generate the alkylating species 6. 6 then undergoes covalent bond formation with a nucleophile (Nu) in the active site leading to irreversible inhibition (Fig. 3). This process finds analogy in the irreversible inhibition of aspartate aminotransferase by serine *o*-sulfate [36].

As implied by its mechanism of action, which demands that it be accepted as a substrate by GABA-T, EOS (5) is a specific irreversible inhibitor of GABA-T having no *in vitro* inhibitory action towards GAD, alanine aminotransferase or aspartate aminotransferase at millimolar concentrations [11]. It apparently does not readily penetrate the brain when administered peripherally, as large doses are needed to influence brain GABA concentrations [37].

More recently, two other enzyme-activated inhibitors of GABA-T, γ-acetylenic GABA (7) [12] and γ-vinyl GABA (8) [13], have been designed and synthesized. With both 7 and 8 the inactivation process has been shown to be time-dependent and irreversible. That the mechanism of inhibition demands that the inhibitors be first accepted as substrates by the enzyme, is confirmed by the demonstration of a primary kinetic isotope effect on the rate of inhibition when the inhibition induced by the γ-deuterio analogs is compared with that of the corresponding protio compounds [38]. This suggests that the inhibition process involves cleavage of the propargylic C–H bond in the case of 7, and of the allylic C–H bond in the case of 8. Both 7 and 8, hence, are accepted as substrates by GABA-T in a similar manner to GABA itself. It is proposed that the inhibition of GABA-T by γ-acetylenic GABA (7) can be rationalized in the following manner (Fig. 4).

Thus the usual abstraction of the proton α to the pyridoxaldimine function followed by reprotonation could lead to the tautomeric imine (path a). In this case the acetylene group, which in 7 is an unreactive appendage to the GABA structure, would enter into conjugation with the newly-generated imine and hence become an alkylating agent, capable of alkylating a nucleophilic residue (Nu) in the active site. Irreversible inhibition would then result from the covalent attachment of

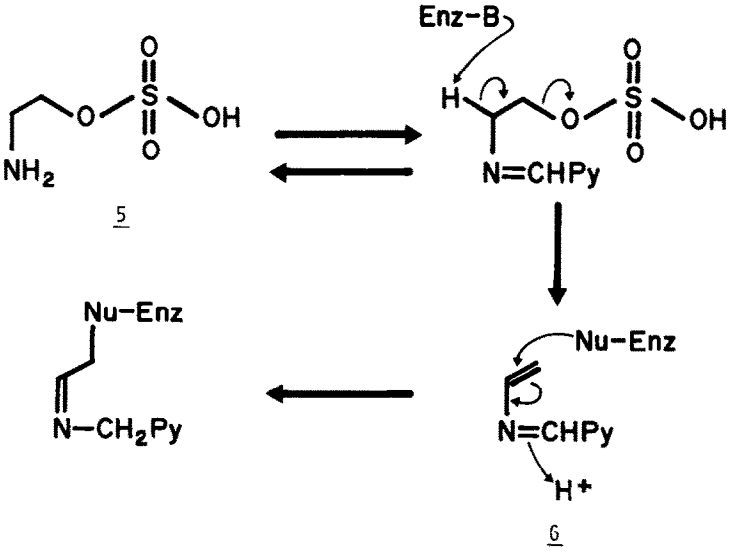

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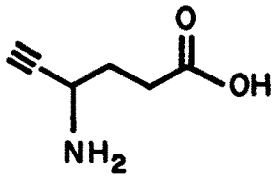
Fig. 3.



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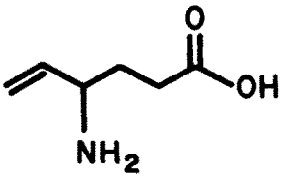

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transformed inhibitor to the active site. Alternatively (path b), the proton abstraction described above could initiate, upon reprontonation, the formation of an allene, which being conjugated to the pyridine ring of the co-enzyme, would also be an active alkylating agent and thus lead to irreversible inhibition [12].

Path a suggests that if the acetylenic group were to be replaced by a vinyl group, i.e. if γ-vinyl GABA (8) is accepted as a substrate by GABA-T, then the initially-unreactive vinyl function would be transformed by the enzyme's own mode of action into an alkylating agent which, once again, could elicit irreversible inhibition [13] (Fig. 5). This proposal is analogous to that originally proposed to account for the irreversible inhibition of aspartate aminotransferase by vinyl glycine [39], although an alternative mechanism involving isomerisation of the double bond of vinyl glycine has now been found to be operative [40] and could also apply to γ-vinyl GABA (8) (Fig. 5).

Both γ-acetylenic GABA (7) and γ-vinyl GABA (8) inhibit GABA-T *in vivo* when administered peripherally [41, 42]. Thus, at a dose of 100 mg/kg i.p. in mice, γ-acetylenic GABA (7) induces a long-lasting inhibition of whole brain GABA-T activity, which drops to nearly zero within 2 hr and is still only 15–20 per cent of control 24 hr after administration. As a consequence brain GABA levels rise to about 4 times control levels at 8 hours, then slowly return to control values [41]. γ-Vinyl GABA (8) enters the brain less readily than does γ-acetylenic GABA (7), hence higher doses are required to induce a comparable inhibition of GABA-T. Thus, a single dose of 1500 mg/kg i.p. in mice reduces brain GABA-T activity to approximately 20 per cent of control, this level of inhibition being maintained for at least 48 hr. Concomitant with this inhibition of GABA-T, brain GABA concentrations rise to about 5 to 6 times control levels, and are still more than double control after 4 days [42].

Both γ-acetylenic GABA (7) and γ-vinyl GABA (8) protect mice against convulsions induced by audiogenic stimuli, electoshock, thiosemicarbazide, isoniazide and strychnine, the pattern of protection mirroring that of whole brain GABA elevation [43, 44]. 7 and 8 do not protect against seizures induced by picrotoxin or bicuculline, suggesting that their anticonvulsant activity is not secondary to their sedative effect [45].

γ-Acetylenic GABA (7), but not γ-vinyl GABA (8), is also an irreversible inhibitor of GAD *in vitro* and *in vivo* [41]. The conception of γ-acetylenic GABA (7) as an irreversible inhibitor of GABA-T was based on its potential acceptance as a substrate by this enzyme. In keeping with this is its observed lack of inhibitory activity towards the PyCHO-dependent alanine and aspartate aminotransferases. Its time-dependent irreversible inactivation of GAD was therefore unexpected. *In vitro* studies using the commercially-available bacterial enzyme suggest that the mechanism of this inhibition can be rationalized in the basis of the microscopic reversibility principle. As γ-acetylenic GABA (7) is an analog of GABA, the product of decarboxylation of glutamic acid catalyzed by GAD, it is believed that the proton abstraction step inherent in the reverse direction

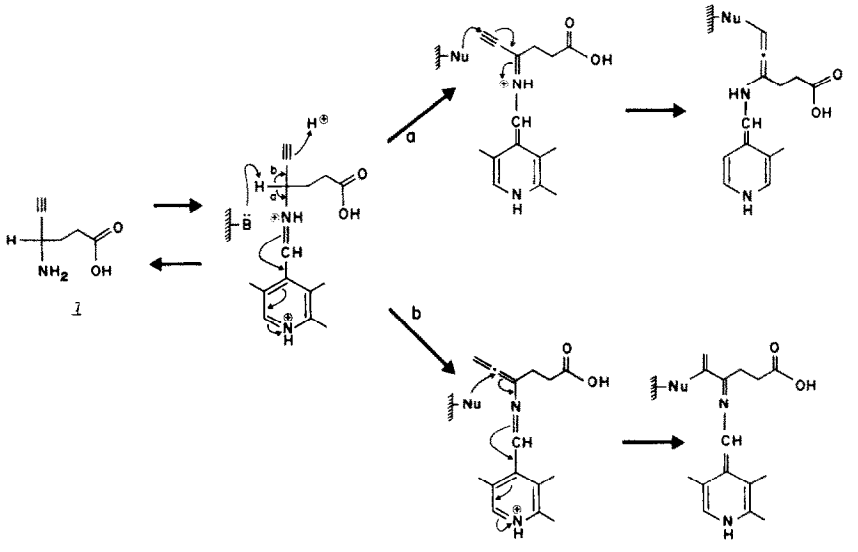


Fig. 4.

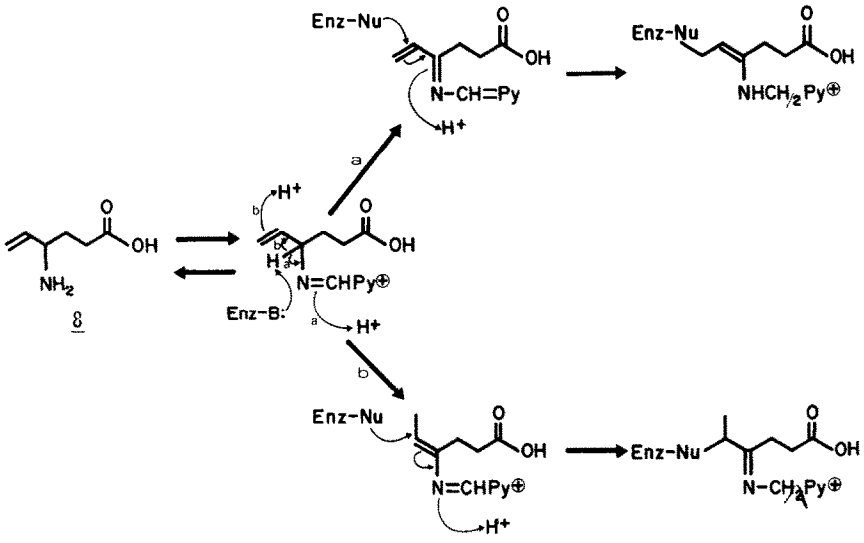


Fig. 5.

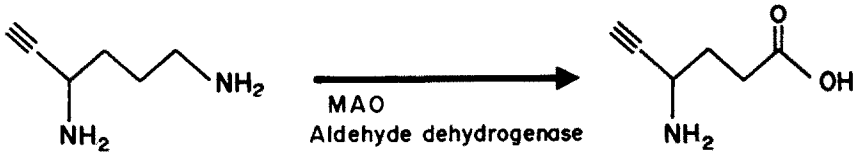
(potential carboxylation of GABA to afford glutamic acid) would initiate, in the case of γ-acetylenic GABA, the formation of a propargylic anion which could lead to irreversible inhibition analogously to that suggested by paths a or b in the inhibition of GABA-T by this compound (Fig. 4) [46].

As a number of PyCHO-dependent decarboxylases operate by a similar mechanism, it was felt that the concept of enzyme inhibition via microreversibility could be generalized. Hence, α-acetylenic putrescine (9) was synthesized and found *in vitro* to be an enzyme-activated irreversible inhibitor of mammalian ornithine decarboxylase (ODC, EC 4.1.1.17), the enzyme which catalyzes the decarboxylation of ornithine to putrescine [47]. On *in vivo* administration to rats, however, not only ODC, but GABA-T and GAD were found to be inactivated, suggesting that α-acetylenic putrescine (9) is metabolized to γ-acetylenic GABA (7) [48]. As the inhibition of GABA-T and GAD *in vivo* by 9 could be prevented by pretreatment with the monoamine oxidase inhibitor, pargyline, it appears that 9 is oxidized via monoamine oxidase in an analogous manner to that found operative for the transformation of monoacetylputrescine to GABA [49].

Recently, γ-amino-β-chloropropionic acid hydroxamide (ACPH, 10) was reported to be an inhibitor of GABA-T, but not of GAD, *in vivo* [50]. A dose of 350 mg/kg, administered intramuscularly, induces a twofold rise in whole brain GABA concentrations as a result of a 48 per cent inhibition of GABA-T. GAD activity was unaffected. No *in vitro* studies were described and speculation as to the mechanism of GABA-T inhibition was not attempted. If, however, the structure of ACPH (10) is compared with that of GABA, it becomes apparent that ACPH is a GABA analog, substituted in the γ position by a chloromethyl group. It is suggested that ACPH will prove to be an enzyme-activated irreversible inhibitor of GABA-T. Thus, if ACPH (10) is accepted as a substrate by GABA-T, the usual proton abstraction α to the pyridoxaldimine function would lead to chloride elimination with the generation of an alkylating species; Irreversible inhibition could then ensure via the condensation of a nucleophilic residue in the active site with this α,β-unsaturated imine (Fig. 6). This proposal finds analogy in the inhibition of alanine racemase by β-chloro-D-alanine [51]. It is thus predictable that other GABA analogs bearing a halomethyl substituent in the γ position will also prove to be irreversible inactivators of GABA-T.

A recent, exciting development is the isolation of a new, potent inhibitor of GABA-T, gabaculine (11) from *Streptomyces toyocaensis* [14]. The inactivation of GABA-T by 11 is time-dependent and irreversible [52, 53], and has been shown to involve a novel enzyme-induced aromatization process [54].

As demonstrated in Fig. 7, gabaculine (11), being a substrate for GABA-T, undergoes the normal Schiff base formation with PyCHO. Proton abstraction, followed by reprotonation, leads to the tautomeric Schiff base, which spontaneously isomerises via a 1,3 hydrogen shift to the *meta*-anthranilic acid derivative 12, the driving force being aromatization of the cyclohexadiene unit. As a result, the cofactor is covalently and irreversibly modified [54].



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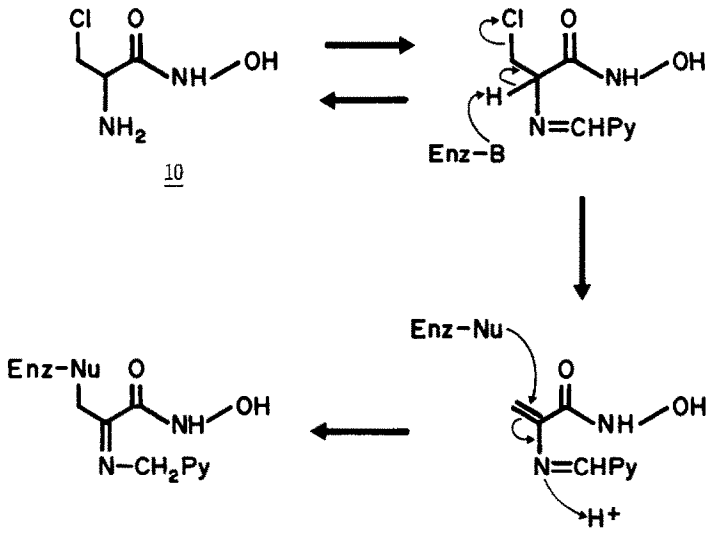


Fig. 6.

Gabaculine irreversibly inhibits GABA-T *in vivo*, when administered by a peripheral route to mice (100 mg/kg, i.p.). Concomitant with this inhibition, brain GABA levels rise until at 20 hr they are 15 times higher than controls [55].

As other cyclohexadienes could be expected to undergo a similar enzyme-induced aromatization, we have synthesized 13, an isomer of gabaculine. As anticipated 13 shows a similar activity *in vitro* and *in vivo* to gabaculine itself [56].

As would be expected from its mechanism of action, gabaculine (11) at millimolar concentrations has no inhibitory effect on GAD, ODC, aspartate aminotransferase and alanine racemase *in vitro* [55]. It has been recently demonstrated, however, that both gabaculine (11) and γ-acetylenic GABA (7), but not γ-vinyl GABA (8), are enzyme-activated irreversible inhibitors of L-ornithine:2-oxoacid aminotransferase *in vitro* and *in vivo* [57]. As a result, not only brain GABA concentrations, but also the level of putrescine is augmented *in vivo* upon administration of 7 or 11 [57].

The design of specific inhibitors of GAD has not yet evolved to the level of sophistication comparable to that of GABA-T inhibitors. Many of the inhibitors in use are non-specific carbonyl trapping agents such as AOAA [19] and glutamic acid γ-hydrazide [24], which also inhibit GABA-T. The convulsant 3-mercapto-propionic acid has been shown to be a competitive inhibitor of GAD [58], 2-mercaptopropionic acid and 2-mercaptoacetic acid being less effective [59]. Thiomalic acid, which more closely resembles glutamic acid is equipotent with 3-mercaptopropionic acid [60]. It has been suggested that the mercapto derivatives inhibit GAD by combining with the pyridoxal phosphate-

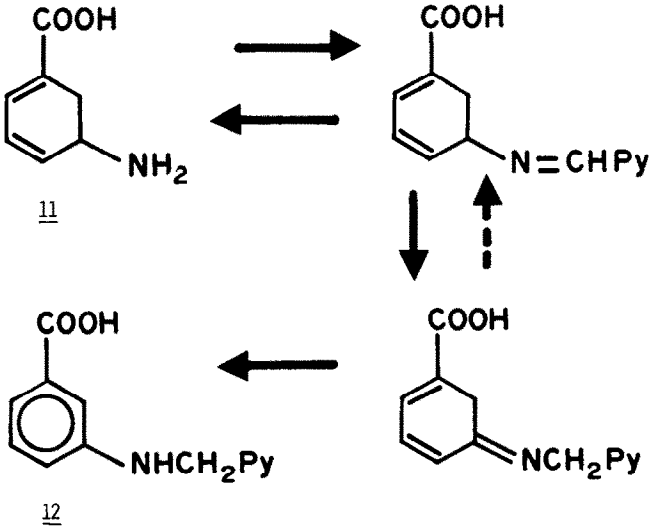
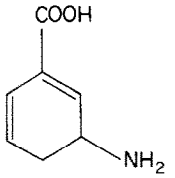


Fig. 7.



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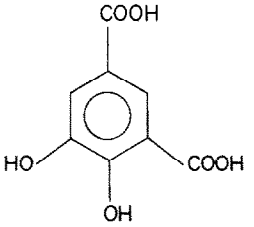
lysine Schiff base. In keeping with this are their inhibitory effects towards GABA-T, although, with thiomalic acid, GABA-T inhibition is less pronounced [60].

Recently, 4,5-dihydroxyisophthalic acid (14), which like gabaculine (11) is elaborated by *Streptomyces toyocaensis* [61], has been found to be the most potent GAD inhibitor known ($K_I = 0.18\mu$M, K_m for L-glutamate = 3.6 mM). Details as to its specificity are as yet unknown.

Allylglycine, long known to be a convulsant [62], demonstrates only weak inhibitory activity towards GAD *in vitro*, and its convulsant activity had been attributed to the formation of a metabolite [63]. This hypothesis has recently found support, as prior treatment of either D- or L-allylglycine with D- or L-amino acid oxidase respectively leads to an oxidation product(s) which powerfully inhibits GAD *in vitro* [64]. That the obvious oxidation product, 2-keto-4-pentenoic acid, or its conjugated isomer 2-keto-3-pentenoic acid, is responsible, has yet to be demonstrated conclusively. Interestingly, the decrease in brain GABA concentrations which occurs after treatment with D-allylglycine, is localized in hind brain areas, in which the preferential localization of D-amino acid oxidase has been reported [65].

CONCLUSION

Several potent and relatively-specific inhibitors of GABA catabolism are now available. Although the clinical utility of GABA-T inhibition is as yet unknown, such specific inhibitors of GABA metabolism will continue to prove useful in the study of the manipulation of GABA-mediated neurotransmission. Undoubtedly, GAD too, will prove to be subject to specific inhibition by an irreversible inhibitor which requires prior transformation by the target enzyme (as distinct from the allylglycine case). Such an inhibitor, although of interest as a pharmacological tool, should also be of use in the mapping of GABA neurons. Finally, it should be appreciated that the experience gained in designing enzyme-activated enzyme inhibitors of GABA-T, as well as the elucidation of the complete mechanism of action of these inhibitors, will prove to be of immense value to the rational design of inhibitors of other enzymes.



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