

Active-Site-Directed Irreversible Inhibition of Rat Brain 4-Aminobutyrate Aminotransferase by Ethanolamine *O*-Sulphate *in vitro* and *in vivo*

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(Received 5 July 1972)

1. Partially purified preparations of rat brain 4-aminobutyrate aminotransferase were inhibited in a time-dependent manner by ethanolamine *O*-sulphate. The inhibition was not reversed by dialysis. 2. The inhibitor formed an initial reversible complex with the enzyme ($K_i = 4.4 \times 10^{-4} \text{ M}$) and the rate of inactivation followed pseudo-first-order kinetics ($k = 7.15 \times 10^{-4} \text{ s}^{-1}$). The inclusion of 4-aminobutyrate markedly slowed the rate of inactivation. 3. Ethanolamine *O*-sulphate did not inhibit glutamate decarboxylase, alanine aminotransferase or aspartate aminotransferase. 4. Intracisternal injection of ethanolamine *O*-sulphate into rats led to rapid inactivation of 4-aminobutyrate aminotransferase *in vivo*.

There is strong evidence that 4-aminobutyrate is an inhibitory neurotransmitter in the mammalian brain (see Krnjević, 1970). The main pathway for its degradation is by the enzyme 4-aminobutyrate aminotransferase (4-aminobutyrate-2-oxoglutarate aminotransferase, EC 2.6.1.19), and one approach to a study of the exact role of 4-aminobutyrate has involved the inhibition of its metabolism. Baxter & Roberts (1961) injected hydroxylamine into rats and reported the inhibition of 4-aminobutyrate aminotransferase activity and increased concentrations of 4-aminobutyrate in the brain. The substituted hydroxylamine amino-oxyacetic acid (Wallach, 1961) is a powerful 4-aminobutyrate aminotransferase inhibitor *in vitro* and *in vivo* and is much used in experiments to block 4-aminobutyrate metabolism. Since the action of these hydroxylamines is not specific in that they inhibit all pyridoxal phosphate-dependent enzymes by formation of oximes with the coenzyme (Roberts & Simonsen, 1963), it must be difficult to conclude with any certainty that physiological and behavioural effects observed on administration of these compounds are due simply to the inhibition of 4-aminobutyrate aminotransferase activity. The objective of the investigations reported in the present paper was the design of a highly specific irreversible inhibitor capable of inactivating 4-aminobutyrate aminotransferase.

Pyridoxal phosphate-dependent enzymes are capable of catalysing different types of reaction, depending on the substrate with which they are presented. For example L-aspartate β -decarboxylase (EC 4.1.1.11) as well as β -decarboxylating L-aspartate also catalyses desulphination of L-cysteinesulphinat, α -decarboxylation of aminomalonate and β -elimina-

tion of β -chloro-L-alanine (Tate *et al.*, 1969). Similarly L-aspartate-2-oxoglutarate aminotransferase (EC 2.6.1.1) catalyses β -elimination of β -chloro-L-glutamate (Manning *et al.*, 1968) and L-serine *O*-sulphate (John & Fasella, 1969), both of which bear a strong resemblance to L-glutamic acid, a normal substrate for the enzyme. Owing to the different reaction pathway followed by these substrates, abnormal intermediates are formed at the active site, and with L-serine *O*-sulphate and aspartate aminotransferase, one of the intermediates, presumed to be aminoacrylic acid, is highly reactive and reacts with a serine or aspartate residue in the active site of the enzyme (John & Fasella, 1969). The enzyme is thus inactivated. A similar inactivation of L-aspartate β -decarboxylase occurs with β -chloro-L-alanine (Tate *et al.*, 1969). These observations suggested a general method for the inactivation of pyridoxal phosphate enzymes which would be highly specific on two counts. First, the substrate should bind well only to those enzymes whose substrates it resembles strongly, and, secondly, inactivation should occur only in those enzymes which catalyse the β -elimination. Ethanolamine *O*-sulphate might be expected to fulfil these requirements and undergo β -elimination catalysed by 4-aminobutyrate aminotransferase, and the reactive aminoethylene produced as intermediate might be expected to inactivate the enzyme.

Materials and Methods

Animals

All rats used were of the Medical Research Council Wistar strain and weighed approx. 200 g.

Chemicals

The coenzymes NAD⁺ and NADH and the enzymes alanine aminotransferase (EC 2.6.1.2), malate dehydrogenase (EC 1.1.1.37) and lactate dehydrogenase (EC 1.1.1.27) were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. Pyridoxal phosphate, 2-oxoglutaric acid and 3,5-diaminobenzoic acid were purchased from Sigma (London) Chemical Co., London S.W.6, U.K. Other chemicals used were supplied by British Drug Houses Ltd., Poole, Dorset, U.K.

Ethanolamine *O*-sulphate was synthesized by the method of Lloyd *et al.* (1962). Succinic semialdehyde was prepared by the method of Bruce *et al.* (1971) and its concentration was assayed by the method of Jakoby (1962) by using preparations of *Pseudomonas fluorescens* as source of succinic semialdehyde dehydrogenase (EC 1.2.1.16) activity. Freeze-dried bacterial extract was obtained from Worthington Biochemicals Ltd. via Cambrian Chemical Co., Croydon, U.K. Protein concentration was determined by the method of Lowry *et al.* (1951).

Assay of enzyme activities

4-Aminobutyrate aminotransferase activity was assayed in the presence of 30mM-4-aminobutyrate, 12.5mM-2-oxoglutarate and 50mM-sodium borate buffer, pH8.4, at 37°C. The amount of succinic semialdehyde produced was determined by the method of Salvador & Albers (1959). The reaction was essentially linear for at least 60min, and routine assays used a 30min incubation time. Enzyme activity was described in units of μmol of succinic semialdehyde produced/h.

Glutamate decarboxylase (EC 4.1.1.15) activity was assayed by the method of Roberts & Simonsen (1963). Aspartate aminotransferase (EC 2.6.1.1) and alanine aminotransferase (EC 2.6.1.2) activities were assayed by using malate dehydrogenase and lactate dehydrogenase respectively (Bergmeyer, 1965).

Experimental and Results

Preparation of rat brain 4-aminobutyrate aminotransferase

The procedure used to obtain a partially purified preparation of rat brain 4-aminobutyrate aminotransferase was based on the initial purification steps described by Vasil'ev & Eremin (1968). The method was modified slightly for use with homogenates of fresh rat brain rather than acetone-dried powder of rat brain.

All operations were performed at 0–5°C unless otherwise stated.

A 20% (w/v) homogenate of freshly dissected rat brain was prepared by using an all-glass homogenizer in a medium containing 10mM-sodium phosphate buffer, pH7.0, 1mM-EDTA, 0.1mM-pyridoxal phosphate, 0.1% 2-mercaptoethanol and 0.5% Triton X-100. The homogenate was centrifuged at 10000g for 60min and the pH of the supernatant was adjusted to pH5.4 with 0.1mM-2-oxoglutaric acid. The acidified solution was then heated at 53°C for 5min and, after cooling in ice, the precipitate was removed by centrifugation at 10000g for 15min. Finely ground solid (NH₄)₂SO₄ was added to the supernatant to give 45% saturation and, after being left to stand at 4°C for 15min, the precipitate was removed by centrifugation at 10000g for 15min. The (NH₄)₂SO₄ concentration in the supernatant was increased to 65% saturation and the precipitated material was isolated by centrifugation and dissolved in the minimum volume of 10mM-sodium phosphate buffer, pH6.5,

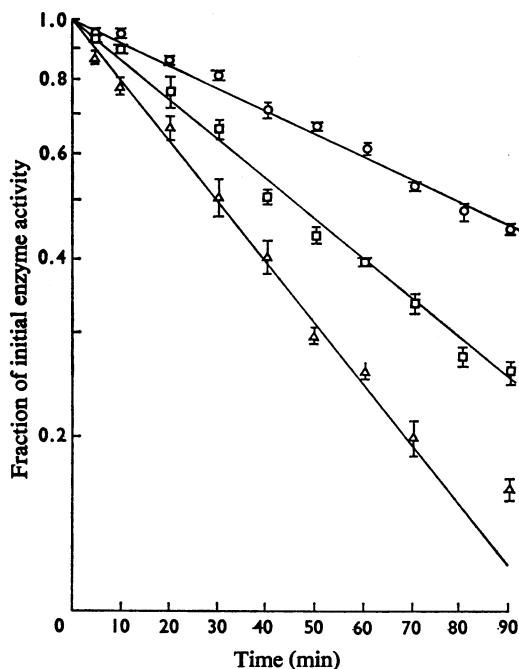


Fig. 1. Time-course of the inactivation of 4-aminobutyrate aminotransferase by ethanolamine *O*-sulphate

The activity at zero time is taken as 100%. The results shown are the mean values of at least four experiments and the vertical bars indicate \pm S.E.M. Concentration of ethanolamine *O*-sulphate: \circ , 0.115mM; \square , 0.25mM; \triangle , 0.5mM. Experimental details are given in the text.

containing 10mM-sodium acetate and 1mM-EDTA. Finally, this solution was dialysed against 100vol. of the same phosphate buffer, pH6.5, for 16h at 4°C with stirring.

By using this procedure it was possible to prepare solutions containing 4-aminobutyrate aminotransferase activity of 10–12 units/mg of protein. The purification achieved was 40–50-fold and the preparations were stable at 4°C for at least several weeks.

Reaction of rat brain 4-aminobutyrate aminotransferase with ethanolamine *O*-sulphate

The inactivation of 4-aminobutyrate aminotransferase by ethanolamine *O*-sulphate was measured by incubating at 37°C the enzyme preparation (approx. 4.0 units/ml) and inhibitor (final concentrations 0.05mM, 0.1mM, 0.115mM, 0.25mM and 0.5mM) in 10mM-sodium phosphate buffer, pH7.0, containing 10mM-sodium acetate, 1mM-EDTA and 0.1% 2-mercaptoethanol. Under these conditions, with no inhibitor present, no loss of enzyme activity occurred during the time-course of the experiments. Portions (50μl) were removed at various time-intervals and pipetted into the normal rate-assay mixtures (total volume 400μl). Preliminary experiments established that the presence of ethanolamine *O*-sulphate at final concentrations of up to 2mM in the rate-assay mixture did not lead to any loss of 4-aminobutyrate aminotransferase activity. The time-course for the inactivation is shown in Fig. 1. These pseudo-first-order plots for the inactivation can be treated by the method of Kitz & Wilson (1962) to give kinetic values for the reaction (Fig. 2). Values obtained were

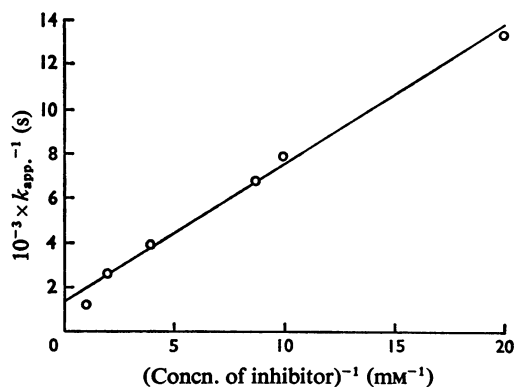


Fig. 2. Dependence of the apparent rate constant of inactivation ($k_{app.}$) on the concentration of ethanolamine *O*-sulphate

Values are plotted as reciprocals as described by Kitz & Wilson (1962).

$K_i = 4.4 \times 10^{-4} M$, and the first-order rate constant for the inactivation, $k_i = 7.15 \times 10^{-4} s^{-1}$.

At the end of the incubation periods shown, the incubation mixture remaining was dialysed against 2 × 100vol. of the 10mM-sodium phosphate buffer, pH7.0, for a total of 16h with stirring. No 4-aminobutyrate aminotransferase activity was restored by this treatment.

Effect of substrate on the inactivation

The effect of 4-aminobutyrate on the inactivation was studied by including 4-aminobutyrate (final concentration 1mM) in an incubation mixture containing 1mM-ethanolamine *O*-sulphate with enzyme preparation at 37°C and pH7.0 as described above. The inactivation process was considerably slowed by the inclusion of 4-aminobutyrate (Fig. 3). This suggests that ethanolamine *O*-sulphate competes with 4-aminobutyrate for binding to the active site of the enzyme.

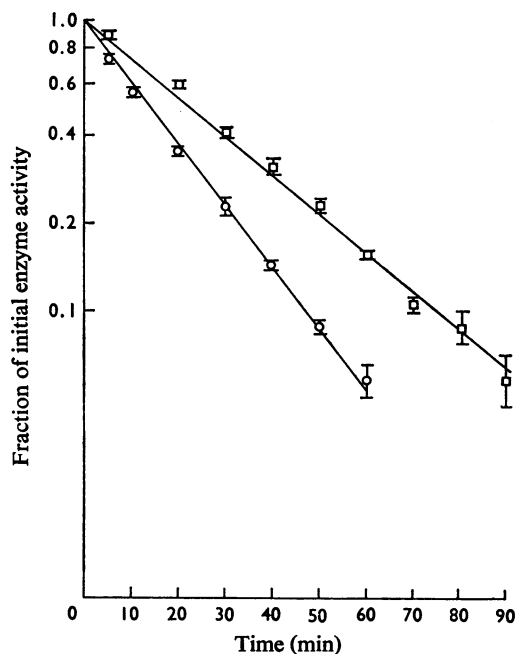


Fig. 3. Time-course of the inactivation of 4-aminobutyrate aminotransferase by ethanolamine *O*-sulphate at 1mM final concentration

○, Inhibitor alone; □, in the presence of 1mM-4-aminobutyrate. Results are shown as mean values of at least four experiments and the vertical bars indicate \pm S.E.M. Experimental details are given in the text.

Effect of ethanolamine O-sulphate on other pyridoxal phosphate enzymes

Ethanolamine *O*-sulphate (1 mM and 100 mM final concentrations) was incubated with pig heart aspartate aminotransferase (prepared by the method of Martinez-Carrion *et al.*, 1967) and alanine aminotransferase for 90 min at 37°C. No diminution of either enzyme activity was observed. Incubation of ethanolamine *O*-sulphate (1 mM, 2 mM and 4 mM final concentrations) with rat brain homogenates for 60 min at 37°C and subsequent assay for glutamate decarboxylase activity showed that this enzyme was not affected by the reagent.

Inhibition of 4-aminobutyrate aminotransferase activity in vivo by ethanolamine O-sulphate

It is known that 4-aminobutyrate does not cross the blood-brain barrier, and in view of the structural similarity it would not be expected that ethanolamine *O*-sulphate would reach the central nervous system if injected intraperitoneally or intravenously. Conse-

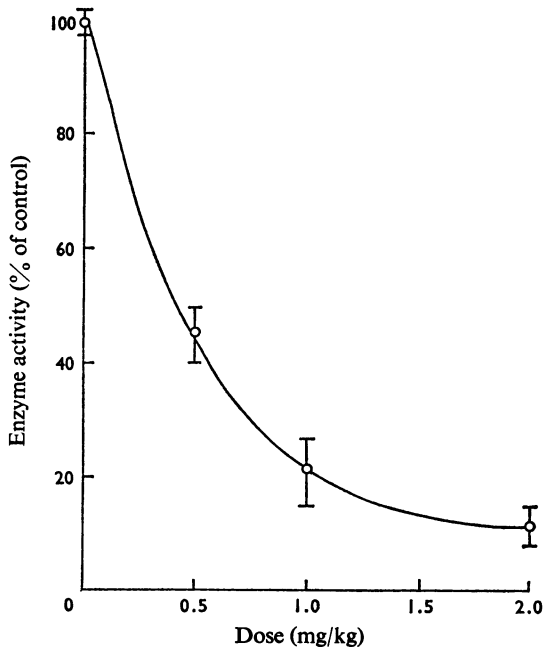


Fig. 4. Effect of intracisternal injection of ethanolamine *O*-sulphate on 4-aminobutyrate aminotransferase activity

Enzyme activity was assayed 24h after injection. Experimental details are given in the text. Each group contained six rats and results are given as mean values \pm s.e.m. The control values are from a group of saline-injected animals.

quently, ethanolamine *O*-sulphate was injected by the intracisternal route into rats to observe its effect on the brain 4-aminobutyrate aminotransferase.

Ethanolamine *O*-sulphate (0.5–2 mg/kg) was injected intracisternally into rats anaesthetized with diethyl ether. Each dose was given to between four and six animals, and control animals were dosed with 0.85% NaCl. After recovery from the anaesthetic, the animals were kept normally and allowed food and water *ad libitum*. After 24 h the animals were killed by decapitation and the brains were removed and 20% (w/v) homogenates prepared in 10 mM-sodium phosphate buffer, pH 7.4, containing 2 mM-2-oxoglutarate, 1 mM-EDTA, 0.1 mM-pyridoxal phosphate, 0.1% 2-mercaptoethanol and 0.5% Triton X-100. The 4-aminobutyrate aminotransferase activity of the homogenates was determined as previously described. Fig. 4 shows the 4-aminobutyrate aminotransferase activity present in the homogenates expressed as a percentage of that present in the saline-injected control animals.

The time-course of the inactivation of 4-aminobutyrate aminotransferase *in vivo* by ethanolamine *O*-sulphate was studied by intracisternal injection of the compound at a dosage of 2 mg/kg; the animals were killed at 1, 2, 4, 6 and 8 h and the 4-aminobutyrate aminotransferase activity of the brain homogenates was determined as described above. Fig. 5 shows the results of these experiments, which indicate that the onset of inactivation is rapid and that

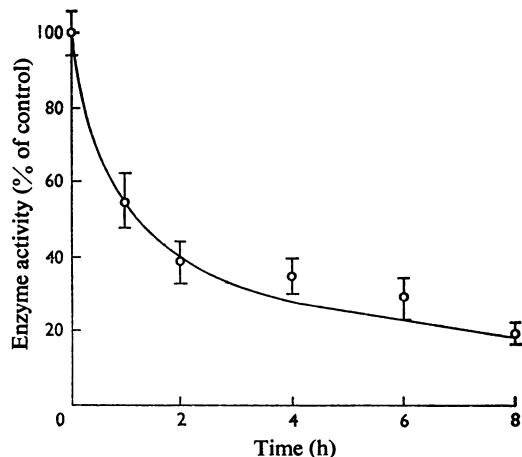


Fig. 5. Time-course of the inactivation of 4-aminobutyrate aminotransferase *in vivo* by the intracisternal injection of ethanolamine *O*-sulphate (2 mg/kg)

Each group contained six rats and the results shown are mean values \pm s.e.m. The control values are from saline-injected animals. Experimental details are given in the text.

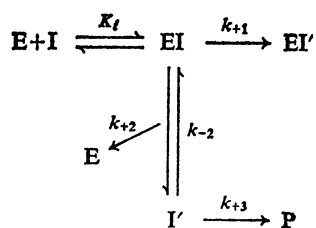
less than 40% of the original activity remains after 2h.

Discussion

The results show conclusively that ethanolamine *O*-sulphate inhibits 4-aminobutyrate aminotransferase activity irreversibly. The fact that the inclusion of 4-aminobutyrate at concentrations in the region of its K_m decreases the rate constant for inactivation by about one-half indicates that inactivation follows an initial reversible binding at the active site. It seems probable that inactivation results from an initial β -elimination of the ethanolamine *O*-sulphate catalysed by the enzyme, but this conclusion is based on analogy with the reaction between aspartate aminotransferase and L-serine *O*-sulphate, since we have not detected the products of β -elimination of ethanolamine *O*-sulphate. It is clear, however, that if the mechanisms of inactivation of the two aminotransferases are the same, the constants governing the initial binding of the sulphate ester and the inactivation process itself must be very different. The K_i value for ethanolamine *O*-sulphate and 4-aminobutyrate aminotransferase is 4.4×10^{-4} M, whereas the K_m value for L-serine *O*-sulphate and aspartate aminotransferase is about 100 times higher. It is noteworthy in this connexion that the affinities of 4-aminobutyrate for 4-aminobutyrate aminotransferase and L-glutamate for aspartate aminotransferase are very similar to the affinities of the respective sulphate esters for these enzymes (Pitts *et al.*, 1965; Jenkins & D'Ari, 1966).

In the case of ethanolamine *O*-sulphate and 4-aminobutyrate aminotransferase the irreversible step is a simple first-order process with a rate constant of $7.15 \times 10^{-4} \text{ s}^{-1}$, whereas the L-serine *O*-sulphate inactivation of aspartate aminotransferase appears to be second order in enzyme concentration. These observations can be reconciled in a relatively simple scheme (Scheme 1). In this scheme, I represents the appropriate sulphate ester, and I' represents aminoacrylic acid where I is L-serine *O*-sulphate and E is aspartate aminotransferase. I' represents aminoethylene where I is ethanolamine *O*-sulphate and E is 4-aminobutyrate aminotransferase. If with L-serine *O*-sulphate and aspartate aminotransferase k_{+2} and k_{-2} are both large compared with k_{+1} and k_{+3} then I will exist at a steady-state concentration which is proportional to [E]. The rate of inactivation will then be apparently second order in enzyme concentration. With ethanolamine *O*-sulphate and 4-aminobutyrate aminotransferase, however, k_{+1} must be much larger than k_{+2} and the scheme becomes a simple reversible binding followed by a first-order inactivation.

4-Aminobutyrate aminotransferase provides the main route for catabolism of 4-aminobutyrate in the



Scheme 1. Reaction of *O*-sulphate substrate analogues with aminotransferases

Proposed scheme to show the pathways that may occur after binding of the *O*-sulphate ester to the enzyme. EI' represents inactivated enzyme. Other details are explained in the text.

brain, so that the irreversible inhibition of this enzyme *in vivo* by ethanolamine *O*-sulphate would appear to provide a useful specific method of studying the physiological effects of increased 4-aminobutyrate concentration.

We are grateful to Mrs. A. Boylett for technical assistance and to Mrs. I. Sutton for performing the glutamate decarboxylase activity assays.

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