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THE ROLE OF 2-KETO-4-PENTENOIC ACID IN SEIZURES INDUCED BY ALLYLGLYCINE

ROGER W. HORTON
Department of Neurology, Institute of Psychiatry,
De Crespigny Park, London, SE5 8AF, England

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Abstract—2 Keto-4-pentenoic acid (2K4PA) was synthesised from L-allylglycine (L-AG) using purified L-amino acid oxidase and isolated relatively free from allylglycine (AG) contamination. 2 keto-4-pentenoic acid is a potent inhibitor of cerebral glutamic acid decarboxylase (GAD) activity, being one to two thousand times more effective than AG. Simultaneous addition of 2K4PA and substrate to GAD gave classical competitive inhibition plots, with a K_i of 10^{-6} M. Preincubation of GAD with 2K4PA for 30 min before addition of substrate produced non-competitive kinetic plots. In the absence of substrate, maximal inhibition of GAD activity was seen within 2–5 min and was only partially reversed by dialysis. The activities of cerebral DOPA decarboxylase, glutamine synthetase and GABA-transaminase *in vitro* were unchanged compared to control activities at concentrations of 2K4PA which produced marked inhibition of GAD activity. *In vivo*, 2K4PA was twenty two and fifty five times more effective than L-allylglycine and D-allylglycine respectively, at inducing seizures in mice after intracerebroventricular injection. The demonstration that 2K4PA is both a more potent convulsant and a more potent GAD inhibitor than the parent compound, AG, is consistent with the metabolic conversion of AG to 2K4PA *in vivo* and rationalises previously observed difference between the effects of AG on GAD activity *in vitro* and *in vivo*.

Allylglycine (AG, 2 amino-4-pentenoic acid) is an amino acid derivative with convulsant properties [1, 2]. The dose of AG required to produce convulsions in 50 per cent of a group of mice is 1.0 m-mole/kg i.p. (115 mg/kg), seizures occurring 2–4 hr after administration of this dose [3]. Studies on the mechanism of the convulsant action of AG have focussed on the decrease in the concentration of brain γ-aminobutyric acid (GABA) following inhibition of brain glutamic acid decarboxylase (GAD, L-glutamate 1-carboxylase, EC. 4.1.1.15 [4]. However, several lines of evidence suggest that a metabolite of AG may be responsible for the inhibition of GAD activity. Such evidence includes:
(1) AG is a weak inhibitor of GAD activity *in vitro* [3] giving rise to nonlinear kinetic plots [3, 4]. The concentration of AG required to produce significant GAD inhibition *in vitro* is an order of magnitude higher than that achieved in the brains of animals treated with a convulsant dose of AG [6, 7].
(2) The inhibition of GAD activity by AG *in vitro* is increased in crude brain homogenates by increasing the duration of the assay or by preincubating the enzyme preparation with AG [5, 8].
(3) Anomalous regional differences in cerebral GAD activity following AG are indicative of a regional variation in the entry or metabolism of AG [9].
(4) The peak brain concentration of AG after i.p. injection is achieved 1–1 hr 30 min before the onset of seizure activity [6, 7].

Recently, it has been shown that both D-allylglycine (D-AG) and L-allylglycine (L-AG) cause seizures in mice, the L form being three to four times as effective as the D form [6]. Both D-AG and L-AG produced very similar weak inhibition of GAD activity *in vitro*. Addition of amino acid oxidase to the *in vitro* GAD assay markedly increased the inhibition of GAD activity [6]. The authors suggested that 2K4PA, formed by the oxidative deamination of AG was the active metabolite responsible for the GAD inhibition.

We report here the preparation and isolation of 2K4PA, its convulsant effect in mice and its potent inhibition of GAD activity.

MATERIALS AND METHODS

Enzymes, co-enzymes and fine chemicals were purchased from Sigma Chemical Co. Other reagents were of the highest grade commercially available. Radiochemicals were purchased from the Radiochemical Centre, Amersham, England.

Swiss S mice were used for *in vivo* experiments and for the preparation of brain homogenates for *in vitro* studies.

Preparation of 2K4PA. 2-keto-4-pentenoic acid was prepared enzymically from L-AG using purified L-amino acid oxidase (L-amino acid: O_2 oxidoreductase (deaminating) EC. 1.4.3.2. from *Crotalus adamanteus* venom) essentially by the method of Collingsworth *et al.* [10]. Incubations were carried out in an atmosphere of 95% O_2/5% CO_2 at 37° for 4–5 hr. Aliquots of the reaction mixture were removed from the incubation at various times, and with appropriate dilution, the absorption spectrum scanned between 210–360 nM, an aliquot reacted with 2,4 dinitrophenylhydrazine and an aliquot added to the GAD assay. In some preparations,

the concentration of AG was determined (as α amino nitrogen) [11]. Concurrent incubations consisting of the complete incubation medium less AG and the complete medium less L-amino acid oxidase and catalase, were used to provide blank samples for the above determinations and for enzyme activity determinations.

Enzyme activities. Glutamic acid decarboxylase activity was estimated by the method of Roberts and Simonsen [12] as modified by Tapia and Awapara [13]. Each ml of reaction mixture contained L-glutamate 32 μmoles, L [1 ^{14}C] glutamate 0.1 μCi; (adjusted to pH 6.3 with KOH), phosphate buffer, pH 6.3, 50 μmoles, dithiothreitol (DTT) 1 μmole, pyridoxal 5′ phosphate, 0.5 μmole and brain homogenate equivalent to 20 mg wet wt tissue. Samples were incubated at 37° for 30 min. Blank preparations and 2K4PA when added, were diluted with 50 mM phosphate buffer pH 6.3, immediately before addition to the assay system. A 10% (w/v) homogenate of brain in 0.2% (v/v) Triton X-100 containing 2.5 mM AET (2-amino ethylisothiouronium bromide hydrobromide) and centrifuged at 3000 g at 4° for 20 min (precipitate discarded) was used as the source of the enzyme.

In kinetic experiments, the basic assay system was unchanged except for varying the concentration of unlabelled L-glutamate between 2–32 μmoles/ml. In some experiments a preincubation period (substrate and enzyme or inhibitor and enzyme) of 30 min at 37° was used.

To study the time course and reversibility of the effect of 2K4PA on GAD activity, a sample of brain homogenate (final concentration 10% w/v) containing DTT (1 μmole/ml), pyridoxal 5′ phosphate (0.5 μmole/ml) and phosphate buffer pH 6.3 (50 μmoles/ml) was incubated at 37° with 2K4PA or a blank extract (control) and samples withdrawn at various times up to 30 min and assayed in the basic assay system. At 30 min 2.5 ml of the incubation medium was dialysed for 3 hr against 800 ml 50 mM phosphate buffer, pH 6.3 containing 1 mM DTT and 0.5 mM pyridoxal phosphate (four changes of medium). Samples were then removed and assayed in the basic assay system.

DOPA decarboxylase (3,4 dihydroxyphenylalanine carboxy-lyase EC. 4.1.1.26) was assayed by the method of Lamprecht and Coyle [14]. The reaction mixture (1.0 ml) contained: L-3,4 dihydroxyphenylalanine 1 μmole (dissolved in water containing 1 mg/ml ascorbic acid), L-3,4 dihydroxyphenyl [1 ^{14}C] alanine 0.1 μCi, phosphate buffer, pH 6.8 75 μmoles, pyridoxal 5′ phosphate 0.1 μmole, EDTA 0.2 μmole, AET 2.5 μmoles and brain homogenate equivalent to 20 mg wet wt tissue. A 10% (w/v) homogenate of brain in 50 mM phosphate buffer, pH 6.8 containing 0.2% Triton X-100 and 2.5 mM AET and centrifuged at 3000 *g* at 4° for 20 min was used as the source of the enzyme.

Glutamine synthetase (L-glutamate: ammonia ligase EC. 6.3.1.2) was assayed by the method of Rowe *et al.* [15]. The reaction mixture (1.0 ml) contained: L-glutamate 50 μmoles, ATP 10 μmoles, $MgCl_2$ 20 μmoles, mercaptoethanol 25 μmoles, imidazole–HCl buffer, pH 6.8, 50 μmoles, hydroxylamine 100 μmoles and brain homogenate equivalent to 20 mg wet wt tissue. A 10% (w/v) homogenate of brain in 0.25 M sucrose was used as the source of the enzyme.

GABA transaminase (4-aminobutyrate-2-oxoglutarate aminotransferase EC 2.6.1.19) was assayed by the method of Waksman *et al.* [16]. The reaction mixture (1.0 ml) contained ketoglutarate 2.5 μmoles, 2-keto [5 ^{14}C] glutaric acid, 0.1 μCi, GABA 5 μmoles, Tris buffer, pH 8.2, 7.0, or 6.5, 50 μmoles and brain homogenate equivalent to 20 mg wet wt tissue. Samples were incubated for 30 min at 37°. A 10% homogenate of brain in 0.2% Triton containing 2.5 mM AET and centrifuged at 3000 *g* at 4° for 20 min was used as the source of the enzyme.

In vivo *experiments.* Groups of mice (six per group) were injected intracerebroventricularly (volume 10–25 μl) under light ether anaesthesia with a range of concentrations of D-AG (150–1140 μg) L-AG (150–507 μg), 2K4PA (8.3–27.9 μg) (pH 6.0) or blank 2K4PA dissolved in 50 mM phosphate pH 6.0 and observed for the onset of seizures. The ED_{50} for the production of convulsions was calculated by the method of Weil [17].

RESULTS

Preparation of 2K4PA. The time course of a typical preparation of 2K4PA is shown in Fig. 1. During the incubation, there was a steady increase in the optical density at 270 nm (E_{270}), a parallel increase in absorption of the 2,4 dinitrophenylhydrazine derivative at 525 nm and an increased ability to inhibit GAD activity *in vitro*. The final isolated product was a yellow viscous oil, with a peak absorption at 270 nM (pH 6.3), which gave a yellow crystalline precipitate with 2,4 dinitrophenylhydrazine. The product was diluted with 0.1 N HCl and stored at

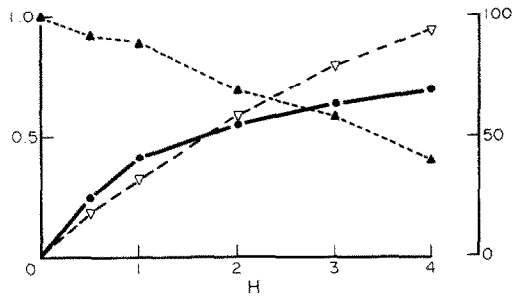

Fig. 1. Time course of a typical preparation of 2-keto-4-pentenoic acid. The initial medium, containing 200 μmoles L-AG, 200 μmoles KCl, 250 μmoles Tris pH 6.6, 5 units L-amino acid oxidase and 5 mg catalase, was incubated in an atmosphere of 95% O_2/5% CO_2 at 37°. Aliquots (50–200 μl) of the reaction mixture were removed at the stated times (and with appropriate dilution) the E_{270} measured ●——● and the E_{525} of the 2,4 di-nitrophenylhydrazine derivative determined (▽- - -▽) shown on the left hand ordinate in extinction units. At the same times an aliquot of the incubation medium was added to the GAD assay (in triplicate) and the mean per cent GAD activity compared to controls (▲- - - -▲) shown on the right hand ordinate. Extinction was compared to concurrent incubations lacking L-AG. Control GAD activity was 28.94 ± 0.56 μmoles/g/hr. The S.E.M. of GAD determinations did not exceed 5 per cent of the mean.

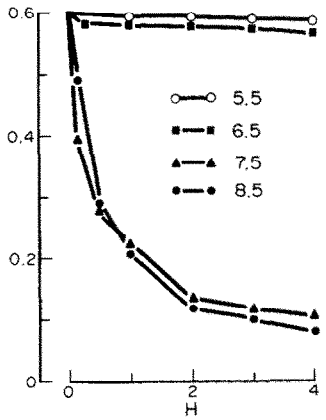

Fig. 2. The effect of time and pH on the E_{270} of 2K4PA. Fresh samples of 2K4PA were diluted to a final concentration of 6×10^{-5} M with 0.1 M phosphate buffer pH 5.5 (○——○), pH 6.5 (■——■), pH 7.5 (▲——▲) and pH 8.5 (●——●) and the E_{270} (ordinate) determined at various times (abscissa) after standing at room temperature. Values are the means of duplicate values.

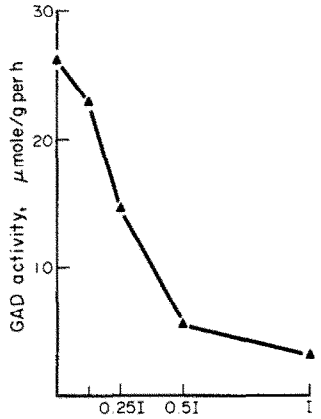

Fig. 3. The effect of 2K4PA on GAD activity *in vitro*. GAD activity (μmoles/g/h) was determined in the absence ($n = 6$) and presence of 2K4PA ($n = 4$ for each concentration). The 2K4PA concentration denoted by I was varied from 5×10^{-6} M to 5×10^{-7} M. Control GAD activity was 27.0 ± 0.4 μmoles/g/hr and the S.E.M. of GAD determinations did not exceed 5 per cent of the mean.

−76°. In two preparations in which the decreases in AG concentration were determined, the overall yield of 2K4PA was 15–20 per cent of the theoretical. A molar extinction coefficient for 2K4PA at 270 nM of 10,000–11,000 was calculated from the decrease in AG concentration, and the figure of 10,000 was used for all subsequent determinations of 2K4PA concentrations. Stock solutions of 2K4PA (15–20 μmoles/ml) were contaminated with 0.3–0.4 μmoles/ml of AG, representing 2–3 per cent of the 2K4PA concentration.

The E_{270} of 2K4PA decreased with time even when the stock solution remained stored at −76°. The half-life of 2K4PA judging from the E_{270} was 5–8 days.

The effect of varying the pH on the E_{270} at room temperature is illustrated in Fig. 2. At pH 5.5 or 6.5 the E_{270} remained fairly constant for up to 4 hr, but at higher pH's there was a marked decrease with time. A sample of 2K4PA treated with 0.1 N excess of NaOH showed a very rapid (less than 3 min) decrease in E_{270}, with a transient new peak at 305 nM which rapidly disappeared. Because of the instability of 2K4PA, each preparation was used only for 5 days and the stock sample was diluted to a standard optical density each day immediately before use.

Effect of 2K4PA on GAD activity. The potent inhibition of GAD activity by 2K4PA *in vitro* is shown in Fig. 3, in which 50 per cent inhibition of GAD activity was produced at a 2K4PA concentration of 1.5×10^{-6} M. Blank samples of 2K4PA (preparations without L-AG or without L-amino acid oxidase and catalase carried through the whole preparation procedure) had no significance on GAD activity at the same dilutions as used for authentic samples of 2K4PA.

The effect of varying the pH of the 2K4PA on its ability to inhibit GAD activity is shown in Fig. 4. At pH 6.5 the inhibition of GAD activity remained constant for up to 4 hr. At pH 8.0 the ability to inhibit GAD activity decreased with time. A sample

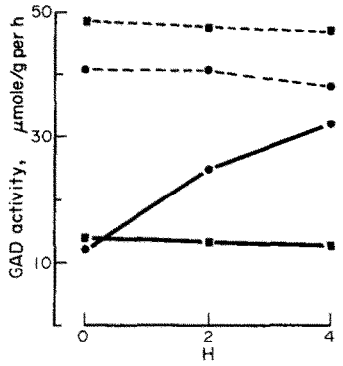

Fig. 4. The effect of pH on the 2K4PA inhibition of GAD activity. Fresh samples of 2K4PA were diluted to a concentration 2.5×10^{-5} M with 50 mM phosphate buffer pH 6.5 or 8.0 at room temperature. Aliquots of the diluted 2K4PA or buffer (control) were added to the GAD assay at various times, ■- - - -■ pH 6.5 control, ■——■ pH 6.5 plus 2K4PA, ●- - - -● pH 8.0 control, ●——● pH 8.0 plus 2K4PA. Values are the mean of triplicate determination, the S.E.M. did not exceed 5 per cent of the mean. The concentration of 2K4PA in the assay mixture was 2.5×10^{-6} M.

of 2K4PA made 0.1 N with respect to NaOH and then adjusted to pH 6.0 had no effect upon GAD activity.

The kinetics of the 2K4PA inhibition was investigated under three conditions. Firstly, the enzyme preparation was added to medium containing substrate and inhibitor. The Lineweaver–Burk plot (I/v v I/s, where v is the enzyme activity and s the substrate concentration) showed a classical competitive inhibition plot, i.e. the inhibition could be overcome by increasing the substrate concentration (Fig. 5). This was confirmed by the Dixon plot (I/v v I, where I is the inhibitor concentration), although there was evidence of nonlinearity at the highest inhibitor concentration used (Fig. 5). A K_i for 2K4PA inhibition of GAD activity was 10^{-6} M (determined graphically from the Dixon plot).

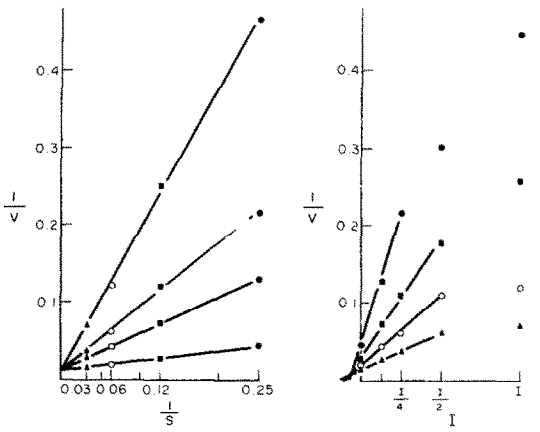

Fig. 5. Kinetic plots of GAD activity in the presence of 2K4PA (inhibitor and substrate added simultaneously). The left hand plot is a Lineweaver–Burk plot of $1/v$ (rate of reaction in μmoles/g/hr) against $1/s$ (the substrate concentration) and the right hand plot is a Dixon plot of $1/v$ against I (where I is the inhibitor concentration). Substrate concentrations were 4 (●), 8 (■), 16 (○) and 32 (▲) mM. Inhibitor concentrations were varied between 10^{-5} and 10^{-7} M. Each point is the mean of triplicate determinations.

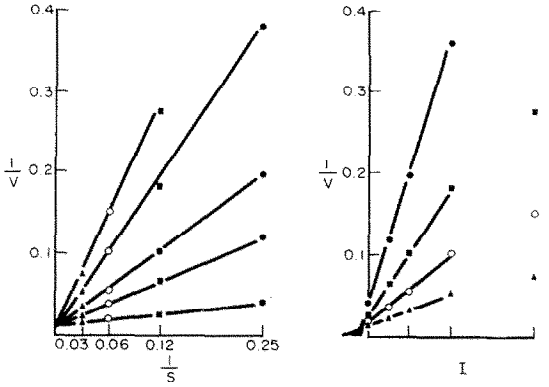

Fig. 6. Kinetic plots of GAD activity in the presence of 2K4PA (enzyme preparation preincubated for 30 min before addition of inhibitors). Remainder of legend as per Fig. 5.

Secondly, the enzyme preparation was preincubated with substrate for 30 min before addition of 2K4PA. The inhibition plots obtained (Fig. 6) and the K_i values were very similar to those obtained without preincubation with substrate (cf. Figs. 5 and 6).

However, preincubation of the enzyme preparation with 2K4PA for 30 min before addition of the substrate produced kinetic plots typical of non-competitive inhibition (Fig. 7). The K_i value from the Dixon plot was 4×10^{-7} M. At the highest inhibitor concentration used deviation from linearity was again observed (Fig. 7 Dixon plot).

The onset and reversibility of the 2K4PA inhibition of GAD activity was investigated by preincubating the enzyme preparation with 2K4PA for various periods of time. At the lower inhibitor concentrations used, maximum inhibition was seen by 5 min and dialysis only partially recovered the enzyme activity (Fig. 8). At the highest inhibitor concentration used, inhibition was maximal within 2 min, was almost complete (less than 5 per cent of the original activity remained) and again was only partially reversed by dialysis (Fig. 8).

Effect of 2K4PA on other cerebral enzyme activities. In order to evaluate the specificity of GAD inhibition by 2K4PA, the activities of cerebral DOPA decarboxylase (another pyridoxal phosphate requiring decarboxylase), glutamine synthetase (for which glutamate is the substrate) and GABA-transaminase (the enzyme involved in the further metabolism of GABA) were determined *in vitro* in the absence and presence of a range of concentrations of 2K4PA.

No significant effect was seen on the activities of any of the three enzymes (Table 1). Because of the demonstrated instability of 2K4PA at pH's above neutrality, GABA-transaminase activities were repeated at pH 6.5, however, the enzyme activity at this pH was too low to measure with accuracy.

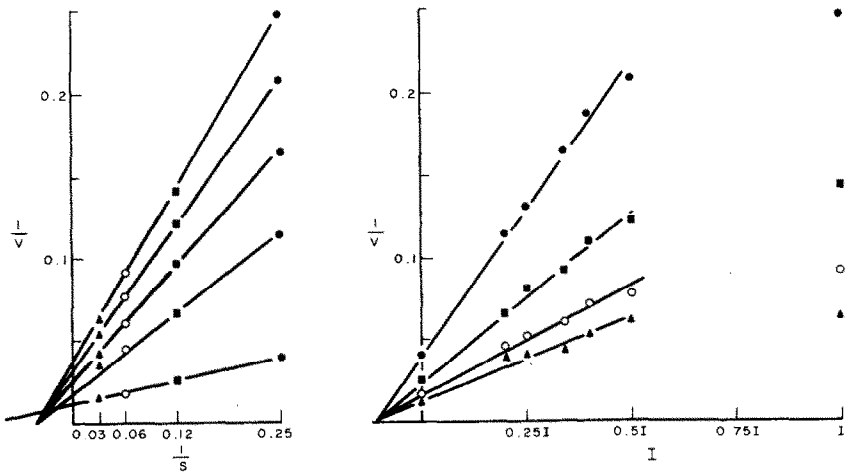

Fig. 7. Kinetic plots of GAD activity in the presence of 2K4PA (enzyme preparation preincubated with inhibitor for 30 min before addition of substrate). Remainder of legend as per Fig. 5, except the inhibitor concentration was varied between 10^{-6} and 10^{-8} M.

Table 1. The effect of 2K4PA on the *in vitro* enzymic activity of GAD, DOPA decarboxylase, glutamine synthetase and GABA-transaminase

	GAD	DOPA decarboxylase	Glutamine synthetase	GABA-transaminase	
				pH 7.5	pH 8.2
CONTROL	27.0 ± 0.4 (6)	2.32 ± 0.08 (6)	13.03 ± 0.58 (4)	28.7 ± 0.9 (4)	64.6 ± 1.9 (4)
5×10^{-5} M 2K4PA	*3.4 ± 0.1 (4)	2.18 ± 0.07 (4)	13.33 ± 0.86 (3)	27.7 ± 0.9 (4)	62.9 ± 1.3 (3)
2.5 ± 10^{-6} M 2K4PA	*6.0 ± 0.2 (4)	2.27 ± 0.04 (4)	13.33 ± 0.86 (3)	—	63.4 ± 1.1 (3)
10^{-6} M 2K4PA	*14.6 ± 0.4 (4)	2.50 ± 0.05 (4)	14.84 ± 0.95 (3)	26.7 ± 0.4 (4)	—
5×10^{-7} M 2K4PA	*23.7 ± 0.3 (4)	2.33 ± 0.06 (4)	—	—	—

Enzymic activities in mouse brain homogenates (see Methods) were determined in the presence of the stated concentrations of 2K4PA or the equivalent volume of buffer (controls). Enzyme activities are expressed as μmoles/g/hr and are presented as the mean ± S.E.M. for the number of determinations shown in parenthesis. Differences between control and 2K4PA activities are denoted by *$P < 0.005$ (Student's 't' test).

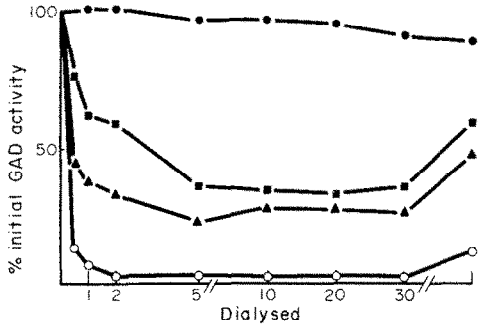

Fig. 8. The time course of the inhibition of GAD activity by 2K4PA *in vitro* in the absence of substrate. The incubation medium contained phosphate buffer pH 6.3 (50 μmoles/ml), DTT (1 μmole/ml) pyridoxal 5' phosphate (0.5 μmole/ml) brain homogenate (10% w/v) and blank extract of 2K4PA (●——●) or varying concentrations of 2K4PA (■——■) 10^{-7} M, ▲——▲ 2.5×10^{-6} M or ○——○ 5×10^{-6} M). Samples were removed at the stated times and added to the basic GAD assay. At 30 min, 2.5 ml of medium was dialysed for 3 hr against 800 ml of original medium (phosphate, DTT, pyridoxal phosphate) with four changes of buffer. Samples were removed and added to the basic GAD assay. Values are presented as the mean per cent of original control activity ($n = 3$). The S.E.M. did not exceed 5 per cent of the mean for any determination.

Effect of intracerebroventricular injection of 2K4PA. Intracerebroventricular administration of 2K4PA in mice produced violent repetitive running convulsions. The ED_{50} for 2K4PA to produce convulsions was 14.5 μg (95 per cent confidence limits, 11.3–18.8 μg) and the mean latency at this dose was 28 min, although some animals convulsed within 10–15 min. No seizure activity was observed in mice administered with the same volume of blank preparations of 2K4PA.

Table 2. The ED_{50} for the production of seizures by D-AG, L-AG, and 2K4PA after intracerebroventricular injection in mice

	ED_{50} for convulsions	95 per cent confidence units
D-AG	804 μg	561–1151 μg
L-AG	375 μg	264–529 μg
2K4PA	14.5 μg	11.3–18.8 μg

Groups of mice (each containing six animals) were injected intracerebroventricularly (10–25 μl) under light ether anaesthesia, with a range of doses of D-AG (115–1140 μg), L-AG (150–507 μg) and 2K4PA (8.3–27.9 μg) and observed for the onset of seizure activity. The ED_{50}'s together with the 95 per cent confidence limits were calculated according to [17].

DISCUSSION

Previous evidence has been suggested that a metabolite of AG, rather than AG *per se*, may be responsible for the inhibition of GAD activity and the convulsions seen after AG administration [3, 5–7, 9]. It has been suggested that 2K4PA is the active metabolite [6]. The present results confirm this hypothesis.

The lack of a direct means of assay of 2K4PA and the lack of authentic samples of 2K4PA for comparison, have necessitated the use of indirect means to quantify its formation and stability. There was a good correlation between the increase E_{270}, and the increase in extinction of the 2,4 dinitrophenyl-hydrazine derivative and the decrease in α-amino nitrogen during the time course of the preparation. This was associated with an increased ability to inhibit GAD activity. Subsequently, we have used the E_{270} as the criterion for quantitative estimation of 2K4PA.

The instability of 2K4PA on storage or under alkaline conditions (as judged by the decrease in E_{270}), was associated with a decreased ability to inhibit GAD activity. The transformation in alkaline solution appears irreversible since reacidification did not restore the E_{270} or the ability to inhibit GAD activity.

The calculated molar extinction coefficient and the spectral properties described for 2K4PA are

similar to those previously reported for 2-oxo-*cis*-4-hexenoic acid, the next higher homologue to 2K4PA [18]. These authors suggested that 2-oxo-*cis*-4-hexenoic acid exists in aqueous solution predominantly in the enol form and that the instability in alkali is due to decarboxylation of the enolate anion [18]. Such an explanation would appear to apply equally well for 2K4PA.

We have demonstrated that 2K4PA (relatively free of AG contamination) is a far more potent inhibitor of GAD activity than the parent compound. Exposure of the enzyme simultaneously to substrate and inhibitor produced classical competitive inhibition plots, suggesting that glutamate and 2K4PA were competing for the same binding site on the enzyme. The deviation from linearity at high inhibitor concentrations may reflect a second lower affinity binding site for 2K4PA, impurities in the sample or self-association of the inhibitor at high concentrations. Preincubation of enzyme and inhibitor produced non-competitive inhibition plots, i.e the K_m for glutamate was unchanged but the rate of reaction was markedly inhibited. The high affinity of the 2K4PA for GAD, combined with only partial reversibility of the inhibition on dialysis suggests that 2K4PA is binding irreversibly (or at least only slowly reversed) to the GAD at a site which does not impair glutamate binding but inhibits subsequent steps in the reaction mechanism. The finding of competitive plots when the enzyme was exposed to substrate and inhibitor simultaneously may reflect a confirmational change in the enzyme in the presence of substrate which restricts the access or binding of 2K4PA. The fact that the onset of inhibition was rapid suggests that 2K4PA is the active metabolite and does not require further metabolism.

It seems likely that the mechanisms of 2K4PA interaction with GAD *in vivo* is probably more fairly reflected by the *in vitro* conditions in which both substrate and inhibitor are present (i.e. competitive inhibition). In this respect it is interesting to compare the convulsant effects of AG with 3-mercaptopropionic acid, a competitive inhibitor of GAD activity [19]. Mercaptopropionic acid induces convulsions with short latency and short duration and generalised onset, with continuous spike and wave EEG activity [3]. This is in marked contrast to the long latency, focal onset and prolonged brief repetitive seizure pattern (up to sixty seizures in 6 hr) with initially full EEG recovery between seizures, seen after AG [3]. These differences in seizure pattern probably reflect differences in the penetration and metabolism of the drugs rather than fundamental mechanistical differences. Such differences include the rapid influx and efflux of mercaptopropionic acid (reflecting the short latency and duration) compared with the comparatively slow but sustained conversion of AG to 2K4PA (reflecting the long latency and duration). The focal onset of seizures after AG may be related to regional variations in the metabolic conversion of AG, a suggestion previously advanced by Fisher and Davies [9].

In vivo, 2K4PA was shown to be a more potent convulsant than either L-AG or D-AG. A similar ratio of the doses of L-AG to D-AG was required to produce convulsions after intercerebroventricular injection as that reported after an i.p. injection [6]. This suggests that the metabolic conversion of AG rather than penetration of AG to the brain is the rate limiting factor in the production of convulsions. This is supported by the finding that the maximum concentration of AG in the brain was observed 1–1 hr 30 min before the appearance of seizures [6, 7].

2 keto-4-pentenoic acid is one to two thousand times as effective as an inhibitor of GAD activity *in vitro* as AG but is only twenty five to fifty five times as effective as L-AG and D-AG respectively at inducing convulsions after intracerebroventricular injection in mice. This apparent discrepancy may be related to the intracerebroventricular route of administration since the doses of D-AG and L-AG required by this route are twenty to thirty times as high as the concentrations of AG achieved in the brains of mice after i.p. administration of convulsant doses of AG. This may reflect a slow absorption of AG from the C.S.F. and limited diffusion within the brain.

Although D-AG and L-AG can be converted to 2K4PA by the respective amino acid oxidases *in vitro*, this may not be the metabolic route of conversion within the brain. The possibility of metabolic conversion of AG to 2K4PA at other sites within the body followed by subsequent penetration into the brain remains to be investigated.

The present demonstration of a potent inhibition of GAD activity *in vitro* and a potent convulsant action of 2K4PA explains the long latency to convulsions after AG administration and rationalises the previously reported discrepancies in the action of AG on GAD activity *in vitro* and *in vivo*. These discrepancies have justifiably prompted several authors to cast doubts on the suitability of AG as a pharmacological agent to study GABA synapses [7, 9]. The present results, including the specificity of the enzymic effects, vindicate the use of AG for such purposes.

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