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Effect of inhibitors of γ-aminobutyrate aminotransferase on the accumulation of ^{3}H-γ-aminobutyric acid by the retina

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Summary

1. Rat retinae pre-incubated and incubated at 37° C in media containing amino-oxyacetic acid (AOAA) (0·1 μM to 1 mM) accumulated more ^{3}H-γ-aminobutyric acid (^{3}H-GABA) than control retinae incubated in the absence of AOAA. This increased accumulation of ^{3}H-GABA by tissue exposed to AOAA was not apparent at short incubation times (0–20 min), but became significant after incubations of 30 min, and maximal after incubation for 60 minutes.

2. At a concentration of 10 μM, AOAA did not alter the apparent K_m for ^{3}H-GABA uptake or V_{max} for either the low or the high affinity GABA uptake systems present in retina.

3. The potentiation of ^{3}H-GABA accumulation produced by AOAA appeared to parallel the inhibitory effect of this compound on 2-oxoglutarate-4-amino-butyrate aminotransferase (GABA-T). Similarly, hydrazinopropionic acid inhibited retinal GABA-T and potentiated the accumulation of ^{3}H-GABA, but hydroxylamine and thiosemicarbazide which did not affect GABA-T, were also without effect on the retinal accumulation of ^{3}H-GABA.

4. *In vitro* incubation with AOAA did not increase the endogenous levels of GABA or other amino acids in the retina.

5. AOAA did not significantly increase the retinal accumulation of radioactive L-glutamate, L-glutamine, taurine, glycine, α-aminoisobutyrate or dopamine: the accumulation of L-aspartate was increased by approximately 30%.

6. The inhibition of retinal GABA-T by AOAA was time-dependent and was not reversed by pyridoxal-5′-phosphate or by repeated washing of the tissue with fresh medium.

7. AOAA also inhibited glutamate decarboxylase (GAD) in retinae incubated *in vitro*. This inhibitory effect was partially reversed by pyridoxal-5′-phosphate.

8. Efflux of radioactivity from the retina was strikingly reduced in the presence of AOAA at concentrations sufficient to inhibit GABA-T by 100%.

9. These findings suggest that AOAA potentiates the accumulation of ^{3}H-GABA by isolated retina, not by increasing the exchange of ^{3}H-GABA with the endogenous GABA pools, but by reducing the metabolism of the amino acid and hence reducing the loss of radioactivity from the tissue in the form of tritiated metabolites.

Introduction

In the mammalian central nervous system, the major degradative pathway for

γ-aminobutyric acid (GABA) is transamination with 2-oxoglutarate to give succinic semialdehyde and glutamate. The transamination of GABA is catalyzed by 2-oxoglutarate-4-aminobutyrate aminotransferase (GABA-T), an enzyme whose precise function in the brain is not altogether clear. It is possible that GABA-T is involved in regulating the storage levels of GABA in the brain, since it has been shown that the parenteral administration of inhibitors of the enzyme produces large increases of brain GABA levels (Baxter & Roberts, 1961). Another mechanism which may be concerned with maintaining GABA levels in the brain is the efficient uptake process for GABA which is present in central nervous tissue ; this uptake process also may be an important mechanism for inactivating the inhibitory actions of GABA on neurones after its release from nerve endings (Iversen & Neal, 1968 ; Krnjević & Schwartz, 1968 ; Curtis, Duggan & Johnston, 1970). Studies of this uptake process using radioactive GABA have shown that despite the presence of GABA-T most of the radioactivity accumulated, i.e. taken up and retained, by central nervous tissue remains as unchanged GABA (Blasberg & Lajtha, 1965 ; Iversen & Neal, 1968 ; Iversen & Johnston, 1971). The possibility that ^{3}H-GABA taken up by the tissue from an external medium enters a pool which is isolated from the largely mitochondrial GABA-T seems unlikely since the subcellular distribution of endogenous GABA and ^{3}H-GABA taken up by brain tissue appear to be identical (Neal & Iversen, 1969). More probably, a proportion of the ^{3}H-GABA taken up by the tissue is catabolized and the radioactive catabolites subsequently diffuse from the tissue into the external incubation medium (Balázs, Machiyama, Hammond, Julian & Richter, 1970 ; Goodchild & Neal, 1973). The present experiments were undertaken to investigate this possibility further by determining the effect of inhibitors of GABA-T on the accumulation of ^{3}H-GABA by central nervous tissue.

Methods

Uptake of ^{3}H-GABA by retina

The uptake of ^{3}H-GABA by the isolated rat retina was measured as described previously (Goodchild & Neal, 1973). Briefly, rat retinae were dissected under ice-cold incubation medium and each was placed into a 25 ml conical flask containing 9·5 ml of ice-cold incubation medium. The retinae were given a preliminary incubation at 37° C for 15 min in a shaking water bath (controls) or pre-incubated for 15 min in the presence of amino-oxyacetic acid (AOAA). ^{3}H-GABA was then added and the incubations were continued for various times. The final concentration of ^{3}H-GABA was 50 nM (0·1 μCi/ml). At the end of the incubation period, the tissue was recovered and washed with ice-cold medium. Each retina was then dissolved in 1·0 ml of Soluene Tm100 (Packard) and the radioactivity was measured by liquid scintillation counting after the addition of 10 ml of phosphor (1% Butyl PBD, Ciba, in toluene).

Efflux of radioactivity from retina

Retinae (approximately 10 mg wet weight) were each given a preliminary incubation in 10 ml of incubation medium for 15 min at 37° C in the presence or absence (controls) of AOAA. ^{3}H-GABA was then added to give a final concentration of 0·1 μM and the incubation was continued for 30 minutes. The tissue was recovered, washed in 5 ml of incubation medium and then each retina was placed in 5 ml of

fresh medium (containing or not containing AOAA, as appropriate). The retinae were incubated in a shaking water bath for 60 minutes. The release of radioactivity was determined by removing 0·2 ml samples from the medium at various times. The incubation volume was kept constant by adding 0·2 ml of fresh medium immediately after sampling. On completion of the incubation, the tissue was recovered and dissolved in 1·0 ml of Soluene Tm100. The radioactivity which remained in the tissue was then estimated by liquid scintillation counting.

Uptake of ^{3}H-GABA by tissue slices

Slices of rat cerebral cortex or spinal cord, 0·5 mm in thickness and approximately 10 mg wet weight, were prepared using a mechanical tissue chopper (McIlwain & Buddle, 1953). The uptake of ^{3}H-GABA by these slices was measured using the same pre-incubation and incubation conditions as those described for retinae.

Estimation of 4-aminobutyrate-2-oxoglutarate aminotransferase (GABA-T) activity

The activity of this enzyme was estimated using the fluorimetric method of Salvador & Albers (1959). Single retinae were homogenized in phosphate buffer (0·01 M) containing disodium edetate (0·67 mM), α-oxoglutarate (17 mM), pyridoxal-5′-phosphate (40 μM), Triton X-100 (0·5% v/v) and β-mercaptoethanol (0·1% v/v) adjusted to pH 7·4 with NaOH (1 M). To each homogenate was added 0·2 ml of a mixture containing α-oxoglutarate (0·1 M) and GABA (0·25 M) in distilled water, and the total volume was made up to 1 ml with borate buffer (0·1 M, pH 8·2). Incubation was carried out at 37° C and halted after 120 min by the addition of 0·1 ml trichloracetic acid (1·52 M). The protein precipitate was removed by centrifugation and 0·1 ml of the supernatant was incubated with 0·1 ml diaminobenzoic acid (0·25 M, adjusted to pH 6·0 with K_2CO_3) at 60° C for 60 minutes. Each sample was then cooled, diluted to 3 ml with distilled water and its fluorescence read at 405/505 mμ in an Aminco–Bowman spectrophotofluorimeter.

The effect of drugs on the enzyme was studied by pre-incubating the retinal homogenates with these compounds for 15 to 60 min at 37° C before adding substrate to start the reaction. Corresponding blanks were prepared by precipitating the enzyme with trichloracetic acid at the beginning of the experiment.

Estimation of L-glutamate-1-carboxylase (GAD) activity

The activity of this enzyme was estimated using the radiochemical method of Roberts & Simonsen (1963). Single retinae were homogenized in 0·8 ml phosphate buffer (0·1 M, pH 6·8) containing Triton X-100 (0·5% v/v) and pyridoxal-5′-phosphate (1 mM) and placed in the main compartment of a Warburg flask. In one side-arm was placed the substrate, 0·1 ml L-glutamic acid-1-^{14}C (1 μCi, 0·1 M) and in the other was placed 0·1 ml H_2SO_4 (1 M). A fluted 1 cm square of Whatman No. 1 filter paper was impregnated with hyamine hydroxide 10-X, allowed to dry and placed in the centre well. The flask was then gassed with N_2 and stoppered. A preliminary incubation was carried out at 37° C for 15 min in a shaking water bath. The substrate was then added and the incubation continued for a further 60 minutes. After this time, the reaction was halted by adding the acid from the other side-arm. The acid also ensured the complete liberation of the $^{14}CO_2$ formed during the incuba-

tion, and this was trapped by the filter paper over the next 30 minutes. The filter paper was then removed and eluted for 30 min in 4 ml ethoxyethanol before adding 10 ml butyl PBD (1% w/v) and estimating the radioactivity by liquid scintillation counting.

The effect of AOAA on the activity of the enzyme was studied by including AOAA in the homogenization mixture. Corresponding blanks were prepared by inactivating the enzyme with the acid at the beginning of the experiment.

Materials

Male and female Wistar albino rats, weighing 150–250 g, were obtained from A. Tuck & Son, Rayleigh, Essex, and used in these experiments.

The incubation medium was Krebs bicarbonate solution of the following composition (g/l.): NaCl 6·92 ; KCl 0·345 ; $CaCl_2$ 0·28 ; $MgSO_4$ 0·144 ; KH_2PO_4 0·162 ; $NaHCO_3$ 2·1 ; D-glucose 2·0. The solution was gassed with 5% carbon dioxide in oxygen to give a final pH of 7·4.

GABA-2,3-^{3}H (2 Ci/mmol) and taurine-1,2-^{14}C (2·5 mCi/mmol) were obtained from New England Nuclear, 6072 Dreieichanhain, West Germany. L-Glutamic acid-U-^{14}C (260 mCi/mmol) ; L-glutamic acid-l-^{14}C (25 mCi/mmol) ; aspartic acid-U-^{14}C (229 mCi/mmol) ; L-glutamine-U-^{14}C (45 mCi/mmol) ; glycine-2-^{3}H (2 Ci/mmol) ; α-aminoisobutyric acid-U-^{3}H (1·8 Ci/mmol) and dopamine-1,2-^{3}H (1·3 Ci/mmol) were obtained from the Radiochemical Centre, Amersham, England. Aminooxyacetic acid (H_2NOCH_2COOH) was obtained from Aldrich Chemical Company Inc., Milwaukee, Wisconsin, hydroxylamine and thiosemicarbazide from British Drug Houses Ltd., hyamine hydroxide 10-X from Intertechnique and pyridoxal-5′-phosphate and β-mercaptoethanol from Sigma Chemical Company. Hydrazinopropionic acid was prepared by the alkaline hydrolysis of 3-pyrazolidone, which was synthesized as described by Van Gelder (1968).

Results

Effect of AOAA on accumulation of ^{3}H-GABA

When individual retinae were incubated at 37° C in medium containing ^{3}H-GABA (50 nM) in the absence (controls) or presence of AOAA there was a rapid accumulation of radioactivity resulting in maximum tissue:medium ratios (dpm/g tissue: dpm/ml of medium) of 50:1 for the controls and 100:1 for retinae exposed to AOAA (10 μM). Previous experiments using identical incubation conditions to the controls and retinae exposed to AOAA indicated that more than 95% of the radioactivity accumulated by the tissue was present as unchanged ^{3}H-GABA (Goodchild & Neal, 1973). Therefore, in the present experiments, the accumulation of ^{3}H-GABA by retinae was measured by determining the accumulation of radioactivity in the tissue.

Effect of AOAA on the time course of ^{3}H-GABA accumulation

The time course of ^{3}H-GABA accumulation in isolated retinae incubated with ^{3}H-GABA (50 nM) at 37° C in the presence or absence (controls) of AOAA is shown in Figure 1. In the control retinae there was an initial phase of rapid uptake (0–10 min) followed by a slower phase of uptake (10–30 min). The maximum accumulation of ^{3}H-GABA occurred after 40 min and a decline in the accumulation

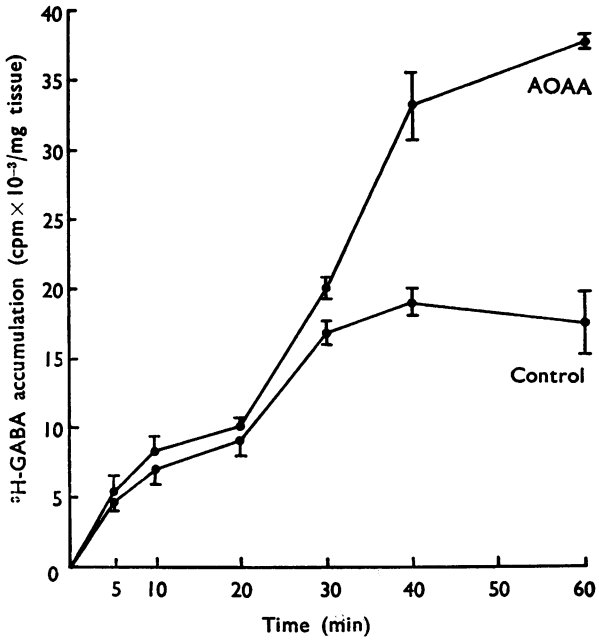

FIG. 1. Time course of ^{3}H-GABA accumulation in retinae incubated at 37° C with ^{3}H-GABA (50 nM) in the absence (controls) and presence of AOAA (10 μM). Each result is the mean of at least six determinations. Standard errors of the means are shown by the vertical bars.

of ^{3}H-GABA occurred when retinae were incubated for longer periods (60 min). In the presence of AOAA (10 μM) the time course of ^{3}H-GABA accumulation was not significantly altered at short incubation times (0–30 min) but after longer incubations (40–60 min) there was a much larger accumulation of ^{3}H-GABA and after 60 min the accumulation of radioactivity in retinae exposed to AOAA was almost double the control values (Fig. 1).

Effect of AOAA on apparent K_m and maximum velocity

The initial rate of uptake of GABA was estimated by incubating retinae at 37° C for 10 min with different concentrations of ^{3}H-GABA (0·1 μM to 1 mM). A double reciprocal plot of the initial velocity of the uptake ((mol/min)/g wet wt. tissue) against the concentration of GABA in the medium revealed two major components (Fig. 2) as described previously (Neal, Peacock & White, 1973). The apparent K_m for the high affinity GABA uptake process in controls was 40 μM and V_{max}= 67 (nmol/min)/g wet wt. tissue: for the low affinity uptake process the apparent K_m=6·5 mM and V_{max}=610 (nmol/min)/g wet wt. tissue. Results obtained with retinae exposed to AOAA (10 μM) were identical to the control values, indicating that AOAA has no effect on the initial velocity of GABA uptake by the isolated retina.

Effect of AOAA concentration on ^{3}H-GABA accumulation

In control experiments the incubation of retinae with ^{3}H-GABA (50 nM) for 60 min at 37° C resulted in tissue:medium ratios of approximately 50:1. The

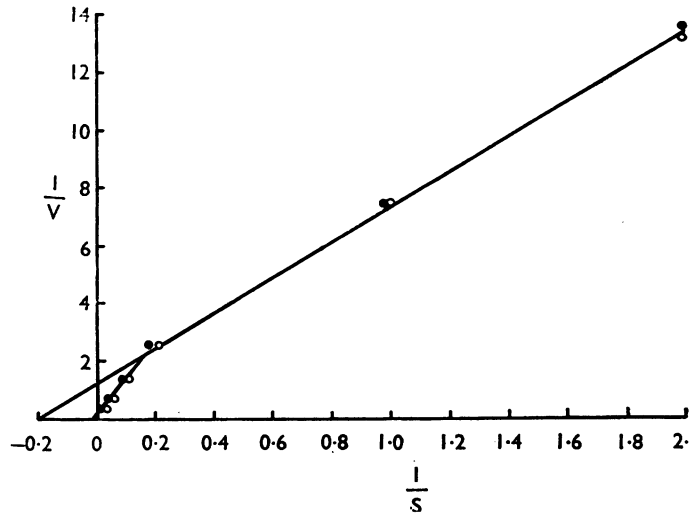

FIG. 2. Kinetic analysis of effect of GABA concentration on rate of ^{3}H-GABA accumulation in absence (controls, open circles) and presence of AOAA (10 μM, solid circles). V=rate of GABA uptake (100 nmol/min)/g wet weight of retina), S=GABA concentration (10 μM). Each value is the mean of at least six experiments.

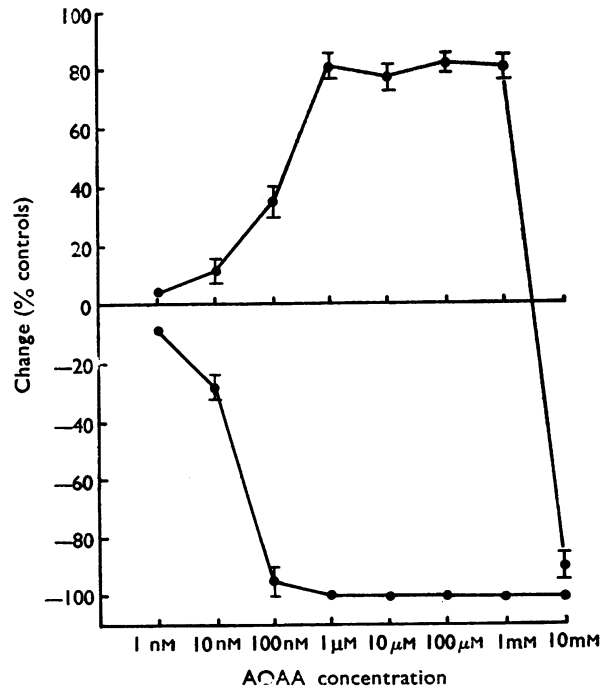

FIG. 3. Upper panel: Effect of different concentrations of AOAA on accumulation of ^{3}H-GABA by retinae incubated at 37° C for 60 min in the presence of ^{3}H-GABA (50 μM). The retinae were preincubated with AOAA for 15 min at 37° C before the addition of ^{3}H-GABA. Lower panel: Effect of different concentrations of AOAA on the activity of GABA-T in retinal homogenates prepared from retinae which had previously been incubated with AOAA for 15 min at 37° C. Each result is the mean of at least six experiments. Standard errors of the means are indicated by the vertical bars.

effect of different concentrations of AOAA on the accumulation of ^{3}H-GABA under these conditions is illustrated in Figure 3. AOAA at concentrations of 1 nM and 10 nM did not significantly increase the accumulation of ^{3}H-GABA but when the concentration of drug was increased to 100 nM, the tissue : medium ratio was increased by 36% compared with controls. When the concentration of AOAA was increased to 1 μM, the accumulation of ^{3}H-GABA was raised by approximately 80%. This increase in accumulation of ^{3}H-GABA was the maximum obtained and higher concentrations of AOAA (10 μM to 1 mM) caused no further increase in the accumulation of radioactivity. At a concentration of 10 mM the effect of AOAA was reversed and there was a marked inhibition of ^{3}H-GABA accumulation (Fig. 3).

The inhibition of GABA-T activity in homogenates of retinae which had been incubated previously for 15 min at 37° C with different concentrations of AOAA was also studied. The activity of GABA-T showed a striking inverse relationship to the potentiation of ^{3}H-GABA accumulation produced by AOAA (Fig. 3). Thus, concentrations of AOAA which had no effect on ^{3}H-GABA accumulation had little inhibitory action on GABA-T. However, AOAA at concentrations which caused a maximum increase in ^{3}H-GABA accumulation inhibited the activity of GABA-T by 100%.

The activity of the GABA-T in retinal homogenates was markedly influenced by the length of time the tissue was exposed to the inhibitor before homogenization. Thus, when retinae were incubated with AOAA for 60 min instead of 15 min before homogenization, the level of inhibition was considerably greater over the lower concentration range. For example, the inhibition of GABA-T activity produced by AOAA (1 nM) was increased from 8% to 69% and at 10 nM the inhibition was increased from 28% to 78%. Furthermore, it was found that after a single exposure of retinae to AOAA (10 μM) for a period of 15 min at 37° C, followed by repeated washing and incubation of the tissues in plain medium for up to 120 min, the usual potentiation of ^{3}H-GABA accumulation still occurred when the tissues were then incubated with ^{3}H-GABA. Also, this potentiating effect of AOAA could not be prevented by including pyridoxal-5′-phosphate (1 mM) in the incubation medium throughout the experiment. These results suggest, therefore, that the inhibition of GABA-T by AOAA is essentially irreversible.

Effect of temperature on accumulation of ^{3}H-GABA

Retinae were pre-incubated for 15 min at 0–4° C in the presence or absence (controls) of AOAA (10 μM). ^{3}H-GABA (50 nM) was then added and the incubation continued for a further 60 minutes. The accumulation of ^{3}H-GABA was greatly reduced in both the controls and in the AOAA-treated retinae. In the controls, a tissue : medium ratio of 2·9 : 1 was obtained. Exposure of the retinae to AOAA (10 μM) potentiated the accumulation of radioactivity, the tissue : medium ratio being increased by 38% ($P<0{\cdot}001$). The activity of GABA-T in the control retinae at 0–4° C was approximately 1% of that at 37° C. This residual enzyme activity was completely inhibited by AOAA.

Effect of other inhibitors of GABA-T on ^{3}H-GABA accumulation

Retinae were pre-incubated for 15 min at 37° C in the presence or absence (controls) of different inhibitors of GABA-T. ^{3}H-GABA (50 nM) was then added

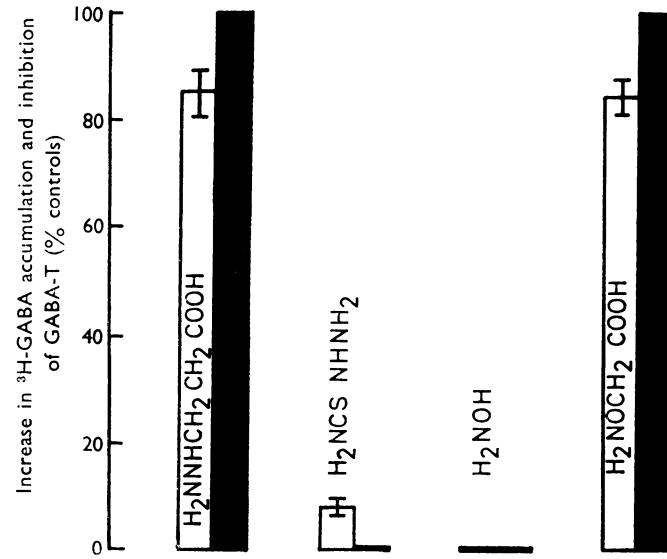

FIG. 4. Effect of various compounds on accumulation of ^{3}H-GABA by retinae (open histograms) and on GABA-T activity in retinal homogenates (solid histograms). For details see Fig. 3. Each result is the mean of at least six experiments. Standard errors of the means are shown by the vertical bars.

and the incubation continued for a further 60 min at 37° C (Fig. 4). Hydrazinopropionic acid (10 μM) completely inhibited the activity of GABA-T and increased the accumulation of ^{3}H-GABA by 80% compared with the controls; these effects were identical to those obtained with AOAA at this concentration (shown by histogram on extreme right of Fig. 4). Thiosemicarbazide (100 μM) and hydroxylamine (100 μM) did not inhibit GABA-T activity and had little or no action on the accumulation of ^{3}H-GABA by the tissue. Increasing the concentration of hydroxylamine to 10 mM reduced the uptake of ^{3}H-GABA to 54% of the control values.

Effect of AOAA on ^{3}H-GABA accumulation by cerebral cortex and spinal cord

The accumulation of radioactivity by slices of rat cerebral cortex and spinal cord after they were incubated with ^{3}H-GABA (50 nM) for 60 min at 37° C is shown

TABLE 1. *Effect of AOAA* (10 μM) *on accumulation of ^{3}H-GABA by retina and slices of rat cerebral cortex and spinal cord incubated at* 37° C *for* 60 *min in the presence of ^{3}H-GABA* (50 nM)

	Tissue radioactivity (dpm/mg)		
	Controls	+AOAA	Increase (%)
Cerebral cortex	12,260±530	18,550±605	51·2
Spinal cord	2,852±184	4,410±220	54·5
Retina	2,929±122	5,468± 72	86·6

The tissues were pre-incubated with AOAA for 15 min at 37° C before the addition of ^{3}H-GABA. Each value is the mean ± S.E.M. of at least six experiments.

in Table 1 together with corresponding values for retina. Uptake was greater in cortex than in cord or retina. When the experiment was repeated in the presence of AOAA (10 μM) after pre-incubating the slices of cortex or cord with AOAA (10 μM) for 15 min at 37° C, tissue levels of radioactivity were obtained which exceeded the controls by approximately 51% and 55% respectively. Thus, the potentiation of ^{3}H-GABA accumulation by AOAA at a concentration of 10 μM and following long incubation periods is not confined to the retina but occurs also in other areas of the central nervous system.

Effect of AOAA on accumulation of other amino acids and dopamine

Retinae were pre-incubated for 15 min at 37° C in the presence or absence (controls) of AOAA (10 μM). Different radioactive amino acids or ^{3}H-dopamine were then added and the incubation continued for a further 60 minutes. The results are summarized in Table 2. AOAA did not influence the accumulation of radioactive glycine, taurine, glutamate, glutamine, α-aminoisobutyric acid or dopamine but the accumulation of ^{14}C-aspartate was significantly increased ($P<0{\cdot}001$).

TABLE 2. *Effect of AOAA* (50 μM) *on accumulation of different amino acids and dopamine by the retina*

Compound	Concentration in medium (μM)	Accumulation of radioactivity (tissue : medium ratio) Controls	+AOAA	Increase (%)
^{14}C-L-glutamic acid	0·35	37·9±1·2	41·4±1·9	9·2 (NS)
^{14}C-L-aspartic acid	0·44	61·5±4·3	83·5±2·6	35·6*
^{14}C-L-glutamine	2·20	27·8±2·3	27·7±2·3	—
^{14}C-taurine	41·00	2·8±0·7	3·1±0·6	11·0 (NS)
^{3}H-glycine	0·05	15·0±0·9	14·6±0·7	—
^{3}H-2-aminoisobutyric acid	0·055	6·7±0·9	6·8±0·8	—
^{3}H-dopamine	0·074	2·9±0·2	2·7±0·2	—

* $P<0{\cdot}001$. NS=not significant. The tissue was pre-incubated for 15 min at 37° C with or without AOAA (controls); radioactive amino acid was then added and the incubation continued for 60 minutes. The accumulation of radioactivity is expressed as the tissue : medium ratio (dpm/g tissue : dpm/ml medium). Each value is the mean ± S.E.M. of six determinations.

Effect of AOAA on endogenous GABA content

Retinae (10 mg wet weight) were incubated at 37° C for 60 min in the absence (controls) or presence of AOAA (10 μM) and then homogenized in 1·0 ml of 0·1 M hydrochloric acid. The homogenates were heated on a boiling water bath for 3 min and then centrifuged to remove the protein. The amino acid composition of each retina was determined by ion-exchange chromatography using an automatic amino acid analyser (Biocal BC100). There was no significant difference in the GABA levels of the controls and retinae exposed to AOAA, the concentrations being 1·72±0·25 μmol/g and 1·64±0·16 μmol/g respectively (mean±S.E.M. of 3 determinations). Similarly, the levels of the other free amino acids which occur in the retina at relatively high concentrations (taurine, aspartic acid, glutamic acid, glutamine and glycine) were not affected by AOAA.

Efflux of radioactivity from retina

Retinae were incubated with ^{3}H-GABA for 30 min in the presence of different concentrations of AOAA or in the absence of AOAA (controls). The retinae were then recovered, washed and transferred to fresh medium containing the appropriate

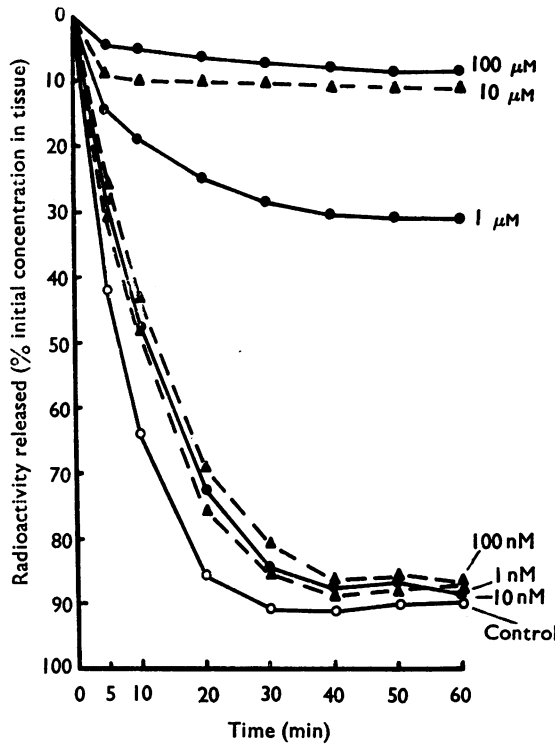

FIG. 5. Efflux of radioactivity from retina. Retinae were incubated with ^{3}H-GABA (100 nM) for 30 min at 37° C in the absence (controls) or presence of different concentrations of AOAA. The tissue was recovered and then placed in fresh medium containing the appropriate concentration of AOAA. The release of radioactivity from the retinae into the medium is expressed as a percentage of the ^{3}H-GABA taken up into the tissue during the 30 min incubation. Each value is the mean of four determinations.

concentration of AOAA and the incubations were continued in the absence of GABA. The efflux of radioactivity from the tissue was estimated by sampling the medium at various times.

The efflux of radioactivity from retinae in the presence of different concentrations of AOAA is shown in Figure 5. In the absence of AOAA, or in the presence of low concentrations of AOAA (1 nM, 10 nM and 100 nM) the efflux curves were virtually identical (Fig. 5). There was an initial rapid efflux (0–10 min) during which about 65% of the radioactivity was lost from the tissue. This was followed by a slower phase of release (10–30 min) and after a total of 30 min incubation, approximately 85% of the radioactivity was lost from the tissue. However, in the following 30 min no further radioactivity passed into the medium. This pattern of release was strikingly altered when the tissue was exposed to higher concentrations of AOAA (10 μM or 100 μM). In the presence of these higher concentrations of AOAA, there was a rapid release of only about 5% of the radioactivity in the tissue (0–5 min) and in the remaining 55 min only a further 9% of the radioactivity was released. Exposure of the tissue to AOAA (1 μM) produced an intermediate pattern of release and after 60 min incubation about 30% of the radioactivity was released from the retinae.

These results indicate that in the presence of AOAA in concentrations which

do not inhibit GABA-T completely (1 nM–100 nM), large amounts of radioactivity are released from the tissue, but when GABA-T is effectively inhibited by higher concentrations of AOAA (10 μM–100 μM), then only a very small release of radioactivity occurs.

Effect of AOAA on GAD activity

Homogenates of retinae were found to possess GAD activity as judged by their ability to liberate $^{14}CO_2$ from L-glutamic acid-l-^{14}C (controls). When the homogenates were pre-incubated with AOAA (1 nM–1 mM) for 15 min at 37° C and then assayed for GAD activity in the presence of added pyridoxal-5′-phosphate (1 mM), a progressively greater inhibition of the enzyme occurred with increasing concentration of AOAA (Table 3). When pyridoxal-5′-phosphate was omitted from the assay mixture, this inhibitory effect was increased in the order of 100 times, indicating there was competition between pyridoxal-5′-phosphate and AOAA for the GAD apoenzyme.

TABLE 3. *Effect of AOAA concentration on GAD activity in retinal homogenates in the presence (+PLP) or absence (−PLP) of added pyridoxal-5′-phosphate* (1 *m*M)

AOAA concentration	Inhibition of GAD (% controls) +PLP	−PLP
1 nM	0	0
10 nM	0	10·8
100 nM	1·1	20·5
1 μM	3·6	62·0
10 μM	23·8	81·8
100 μM	75·7	87·2
1 mM	97·5	96·3

Homogenates were pre-incubated for 15 min at 37° C with or without AOAA (controls) before assaying GAD activity. Results shown are the means of two experiments and are expressed as a percentage of the GAD activity in controls.

Discussion

The present study shows that AOAA and some other inhibitors of GABA-T significantly potentiate the accumulation of ^{3}H-GABA by the retina, when the tissue is incubated *in vitro* for 30 min or longer. The increased ^{3}H-GABA accumulation by tissues exposed to AOAA compared with controls appears to be closely related to the inhibition of GABA-T produced by this compound, since (1) the increase in ^{3}H-GABA accumulation with increasing concentration of AOAA closely parallels the inhibition of GABA-T activity in the tissue ; (2) concentrations of AOAA higher than those necessary to inhibit GABA-T by 100% do not cause any further potentiation of ^{3}H-GABA accumulation by the retina ; (3) hydrazinopropionic acid at a concentration which inhibited GABA-T by 100% produced the same degree of potentiation of ^{3}H-GABA accumulation as AOAA. On the other hand, hydroxylamine and thiosemicarbazide, which in the present experiments did not inhibit GABA-T, did not potentiate the accumulation of ^{3}H-GABA ; (4) the endogenous GABA levels of isolated retinae were not affected by concentrations of AOAA sufficient to inhibit GABA-T by 100%. Therefore, it seems unlikely that this compound potentiates the accumulation of ^{3}H-GABA by increasing the exchange of radioactive amino acid with the endogenous GABA pools ; (5) AOAA did not affect the transport process for GABA directly since the apparent K_m and V_{max} were apparently unaltered in the presence of AOAA at a concentration which produced maximum potentiation of ^{3}H-GABA accumulation ; (6) large effluxes of radio-

activity occurred from control retinae and retinae which had been exposed to AOAA in concentrations insufficient to inhibit GABA-T. However, when the tissue was exposed to higher concentrations of AOAA which completely inhibited GABA-T, then the efflux of radioactivity was extremely small.

These results are consistent with the suggestion that control retinae and retinae exposed to AOAA both take up ^{3}H-GABA at the same rate, but that in the controls the ^{3}H-GABA is eventually catabolized to radioactive products which are rapidly lost from the tissue. This model is supported by previous efflux studies of the retina which showed that the large amounts of radioactivity released from control retinae consisted mainly of acidic and neutral metabolites, whilst the much smaller amounts of radioactivity released from retinae exposed to AOAA consisted mainly of unchanged ^{3}H-GABA and only small amounts of acidic and neutral metabolites (Goodchild & Neal, 1973). Other studies have also shown that GABA is strongly retained by brain tissue *in vitro* compared with its catabolic products (Balázs *et al.*, 1970). The above results may explain why the maximal uptake of ^{3}H-GABA by retina or by cortical slices is greater at 25° C than at 37° C: the apparent K_m for the GABA uptake process is approximately the same at these two temperatures (Starr, unpublished results), but at the higher temperature the catabolism of ^{3}H-GABA would be greater than at 25° C and larger amounts of radioactive products might be expected to be lost from the tissue.

The effect of AOAA on ^{3}H-GABA accumulation by the retina was remarkably specific since the compound did not influence the accumulation of radioactive glycine, taurine, L-glutamine, L-glutamic acid, α-aminoisobutyric acid, or dopamine; the accumulation of L-aspartic acid, however, was increased by 30% compared with controls, presumably due to inhibition of aspartate aminotransferase.

AOAA at a concentration which inhibited GABA-T by 100% (10 μM), did not significantly alter the endogenous levels of glutamate, aspartate, taurine, glutamine, glycine, or GABA. The failure of AOAA to increase the tissue levels of GABA, a conspicuous action of this drug *in vivo* (Baxter & Roberts, 1961), is explained by the inhibitory action this compound was found to have on glutamic decarboxylase (GAD), the enzyme responsible for GABA formation. The present study indicated that in the retina, under the *in vitro* incubation conditions used, AOAA (10 μM) inhibited the action of GAD by approximately 80%. This inhibitory effect of AOAA on retinal GAD, unlike that on GABA-T, was antagonized to some extent by pyridoxal-5'-phosphate. These results confirm similar studies on the effects of carbonyl trapping agents on GAD and GABA-T (Baxter & Roberts, 1961). It has been suggested that the reversal by pyridoxal-5'-phosphate of the inhibitory action of AOAA on GAD, but not GABA-T, indicates a less firm binding of the coenzyme to GAD than to GABA-T (Baxter & Roberts, 1958; 1961).

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