Biochemical Pharmacology, 1966, Vol. 15, pp. 533–539. Pergamon Press Ltd., Printed in Great Britain.

THE EFFECT OF AMINOOXYACETIC ACID ON THE METABOLISM OF γ-AMINOBUTYRIC ACID IN BRAIN

NICO M. VAN GELDER

Department of Pharmacology, Tufts University School of Medicine,
Boston, Mass. U.S.A.

(*Received* 30 *September* 1965; *accepted* 18 *November* 1965)

Abstract—With the aid of histochemical technique that is specific in demonstrating γ-aminobutyric acid (GABA) metabolism, aminooxyacetic acid was shown to cause strong inhibition *in vivo* of GABA-α-ketoglutarate transaminase which was not reversed by GABA. Succinic semialdehyde dehydrogenase was not affected by this compound. With the rates of formazan production in brain sections as a basis for comparison, the results showed that GABA metabolism was much slower than succinic semialdehyde oxidation, indicating that *in vivo* transamination is probably rate limiting with respect to GABA degradation.

After maximal inhibition of GABA transaminase activity by aminooxyacetic acid, GABA synthesis *in vivo* was shown to be 5–6 μmoles/g per hour. The maximal brain GABA levels that could be reached *in vivo* under these circumstances were 20 μmoles/g fresh tissue.

Finally, administration of GABA to animals which had received aminooxyacetic acid either enhanced or caused severe impairment of motor function. Although the symptoms were compatible with a central action of GABA, no evidence could be obtained that exogenous GABA penetrated into the central nervous system under these conditions.

IN 1961 Wallach[1] reported that aminooxyacetic acid was a competitive inhibitor of GABA–α–ketoglutarate transaminase *in vitro*. He also showed that after administration of this compound brain GABA levels rose. The development of a new histochemical technique which specifically demonstrates GABA metabolism[2] afforded an opportunity to use aminooxyacetic acid to obtain more information regarding the metabolism of GABA *in vivo*.

In this paper evidence is presented which shows that the rise in GABA levels that occurs after aminooxyacetic acid administration can be correlated with an inhibition *in vivo* of GABA transaminase and that transamination is probably the rate-limiting step in GABA metabolism. In addition it was possible to obtain an estimate of the maximal rate of GABA synthesis and to show that an increase in GABA levels *in vivo* is probably limited by the amount of GABA that can be bound in brain.

The present work also describes a pharmacological action of GABA which was observed only after aminooxyacetic acid had been administered. No evidence could be obtained that this action was due to a central effect of GABA despite the fact that the symptoms were compatible with such an explanation.

METHODS

Histochemistry. The conditions for demonstrating GABA metabolism have been described in detail.[2] The method consists briefly of incubating cold stored (−18°) frozen sections at 40° in contact with an agar–saline gel (pH 7·4) which has the following composition: Nitro BT*, 2 mg/ml; GABA, 5 mg/ml; α-ketoglutarate, 5 mg/ml; NAD, 2 mg/ml. The resulting reduction of Nitro BT to the insoluble formazan form reflects GABA metabolism. For the demonstration of succinic semialdehyde dehydrogenase, succinic semialdehyde was substituted for GABA. This compound was obtained from Chemicals Procurement Laboratories as a dimer which was converted to the monomer by acid hydrolysis (pH 2, 30 min, 100°).

Control sections were incubated under the same conditions in an identical medium from which the substrate, GABA or succinic semialdehyde, had been omitted. As reported previously,[2] such sections exhibited no formazan staining.

Injections. Aminooxyacetic acid (K & K Laboratories), 20–40 mg/kg, was administered s.c. to male mice (25–30 g), male guinea pigs (250–300 g), and male rabbits (2–3 kg). At a variable time after this injection GABA was administered i.p. (500 mg/kg) to mice and guinea pigs; rabbits received GABA i.v. (20 mg/kg). In control animals saline injections were substituted for either aminooxyacetic acid or GABA. The concentrations of the compounds were adjusted so that the animals never received more than 0·2–0·4 ml per injection.

GABA measurements in mouse brain. Mice were killed by severing the cervical vertebrae. The brains (minus cerebellum and brain stem) were removed and dropped into a beaker of petroleum ether kept at −70° by a dry ice–ethanol bath. The time between death and freezing of the brain was 25 to 35 sec. The brains were rapidly blotted, weighed, and dropped into 1·5 ml of 0·1 N HCl kept at 100° in a water bath. After 10 min, they were homogenized and the homogenates were returned for another 10 min to the water bath. The solutions were then neutralized with 2 drops of 1 N NaOH and centrifuged. An aliquot, usually 25–50 μliters, of the supernatant fluid was assayed for GABA by the enzymic method of Jakoby and Scott.[3]

RESULTS

Approximately 10 min after animals had been injected with aminooxyacetic acid they began to show unsteadiness and staggering, and they occasionally suffered a few convulsions which were rarely fatal. With time, such animals appeared to develop muscular weakness which finally resulted in an inability to support themselves. The severity of the symptoms depended both on the dose of aminooxyacetic acid and on the species. Guinea pigs appeared to be particularly sensitive to the effects of aminooxyacetic acid, and in these animals 20 mg/kg was sufficient to cause complete motor disability. Mice and especially rabbits were much less responsive even when the dose of aminooxyacetic acid was increased to 40 mg/kg. This difference in sensitivity between species was also observed by Wallach.[1]

When GABA was injected into animals treated in the above manner with aminooxyacetic acid, the impairment of motor function, which until then might have been minimal, became more pronounced. The action of GABA was most dramatic in rabbits, and in Fig. 1 an attempt has been made to demonstrate this effect.

* Nitro BT: 2,2′-di-*p*-nitrophenyl-5,5′-diphenyl-3,3(3,3′-dimethoxy-4,4′-biphenylene)-ditetrazolium chloride.

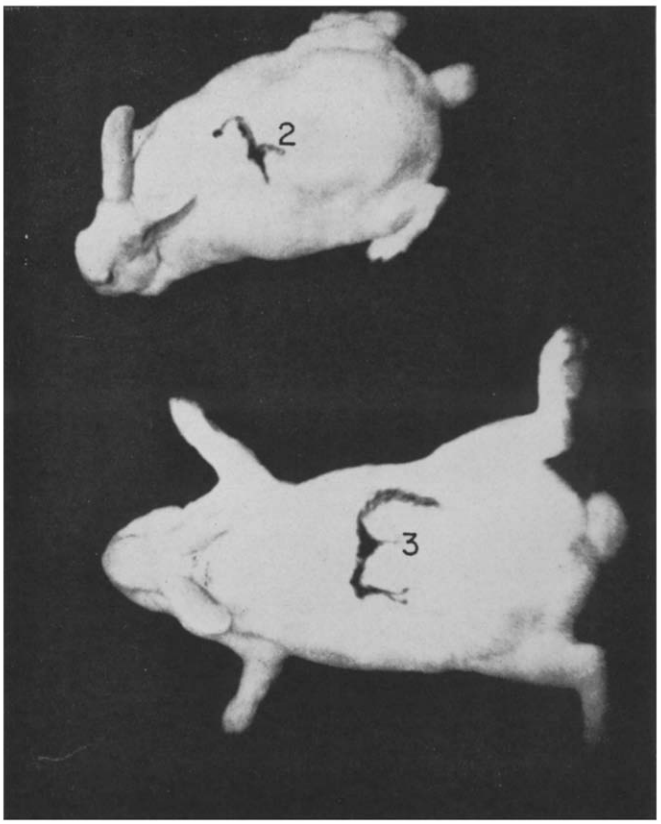

FIG. 1. The action of intravenous GABA after injection of aminooxyacetic acid. Rabbit 2 received 40 mg aminooxyacetic acid/kg s.c. 90 min earlier. Rabbit 3 received the same dose of aminooxyacetic acid at the same time but in addition received 20 mg GABA/kg i.v. 30 min before the photograph was taken. Note difference in position of the legs (animals seen from above). Rabbits which received as much as 500 mg GABA/kg alone showed no symptoms.

facing page 534

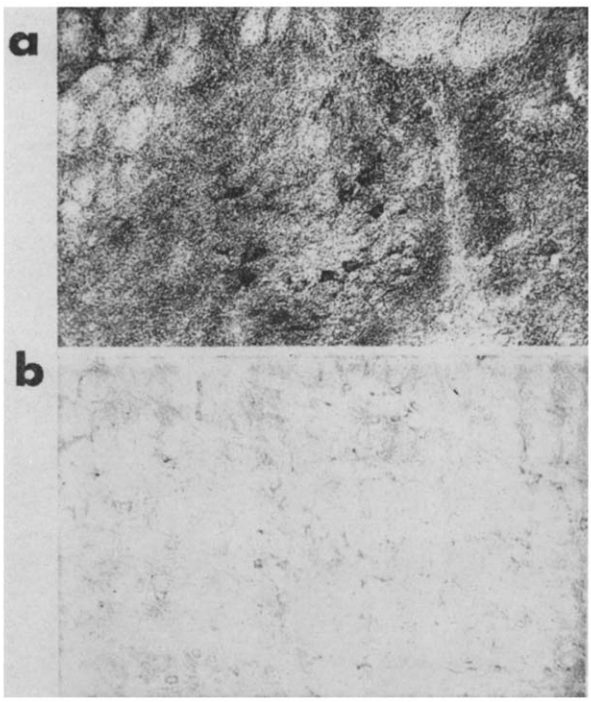

FIG. 2. The effect of aminooxyacetic acid on GABA metabolism.

(a) Section from mouse injected with saline and incubated in a normal medium.

(b) Section from mouse injected with 20 mg aminooxyacetic acid/kg 4 hr previously and incubated in the same medium.

Sections are through the brain stem, showing hypoglossal nucleus. Unstained regions are white matter that exhibits no transaminase activity;[2] 100× magnification.

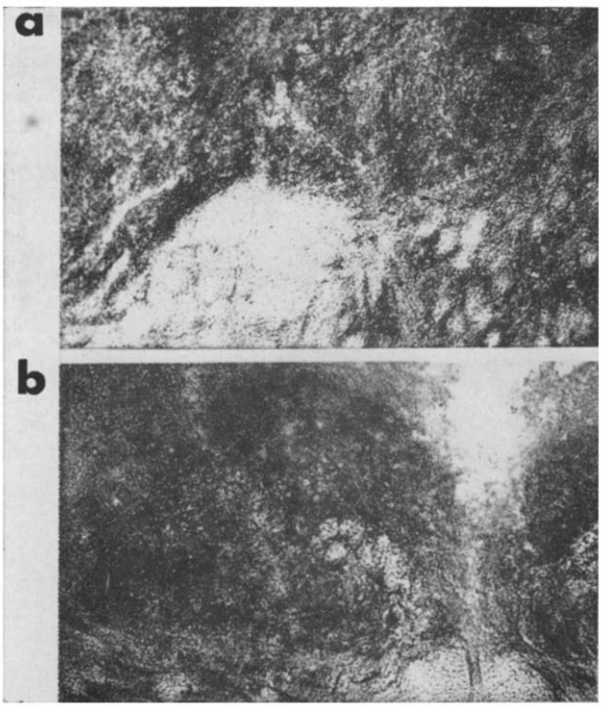

FIG. 3. Formazan production with succinic semialdehyde as substrate.

(a) Section from mouse injected with saline and incubated in a medium containing succinic semialdehyde.

(b) Section from mouse injected with 20 mg aminooxyacetic acid/kg 4 hr previously and incubated in the same medium.

Same region as shown in Fig. 1; × 100 magnification.

It is apparent from this photograph that the rabbit which received both GABA and aminooxyacetic acid (No. 3) became completely ataxic. The animal had received an i.v. injection of 20 mg GABA/kg 1 hr after a s.c. injection of 40 mg aminooxyacetic acid/kg. When placed on its back this rabbit would not right itself, although it seemed fully conscious. The signs appeared gradually over a period of 30 min after the injection of GABA and persisted for at least 2 hr; recovery was not complete until 8–10 hr later. In contrast, the other rabbit (No. 2) which received aminooxyacetic acid alone showed only mild motor incoordination and weakness as indicated by the position of its hindlegs. In the course of several hours no further impairment was obvious. Other rabbits which were injected only with GABA never showed any abnormalities even at doses as high as 500 mg/kg. The observed effects of GABA could therefore not have been due to a peripheral action of GABA. On the contrary, they closely resembled the action or GABA when it was injected into the ventricles of cats (unpublished Ph.D. thesis, 1959).

Similar experiments with guinea pigs were more difficult to evaluate since in these animals doses of aminooxyacetic acid as low at 15–20 mg/kg were sufficient to cause complete impairment of motor function approximately 20 min after injection. Subsequent i.p. injections of GABA (500 mg/kg) occasionally appeared to be the cause of death, which may have been due to respiratory failure. In mice, i.p. injections of 500 mg GABA/kg after aminooxyacetic acid (40 mg/kg) sometimes seemed to enhance motor function deficits but sometimes appeared to have no effect.

The development of a method which is specific in demonstrating GABA metabolism in brain tissue[2, 4] afforded an opportunity to determine whether the motor impairment in aminooxyacetic acid-treated animals could be correlated in any way with an inhibition of GABA metabolism *in vivo*. The results presented in Figs. 2 and 3 are typical examples of such studies. The section in Fig. 2a was from a mouse injected with saline and shows the strong formazan production that occurs after incubation with GABA (see Methods). As usual,[2] no formazan formation occurred in comparable sections incubated in an identical medium from which GABA had been omitted. The section shown in Fig. 2b was obtained from a mouse which had received 20 mg aminooxyacetic acid/kg 4 hr previously. The incubation medium was the same as that used for the section shown in Fig. 2a. Since formazan production is a reflection of GABA metabolism, Fig. 2b shows that such metabolism was practically abolished after a dose of 20 mg aminooxyacetic acid/kg. The brain stem sections in Fig. 3 were obtained from the same two mice, but the incubation medium contained succinic semialdehyde instead of GABA. It is obvious that brain tissues of both animals were equally capable of metabolizing succinic semialdehyde, as evidenced by the strong formazan precipitate in Figs. 3a and 3b. The contrast between Figs. 2b and 3b shows that the inhibition of formazan production by aminooxyacetic acid was due specifically to inhibition of GABA–α–ketoglutarate transaminase and that succinic semialdehyde dehydrogenase was not affected.

Several other points became apparent in experiments of this type. In comparing Fig. 2a and Fig. 3a, it is seen that the section incubated with succinic semialdehyde is darker and somewhat more extensively stained. This was observed every time sections were incubated with succinic semialdehyde instead of GABA. Also, the rate of formazan production was much faster in such sections, and the reaction was usually complete in approximately 5 min rather than the usual 15 min in the presence of

GABA. Control experiments similar to those carried out previously for GABA[2] showed that formazan production in the presence of succinic semialdehyde was dependent on enzyme activity. Another difference found was that the presence of α-ketoglutarate in the incubation medium had an inhibitory effect on the oxidation of succinic semialdehyde but not of GABA.

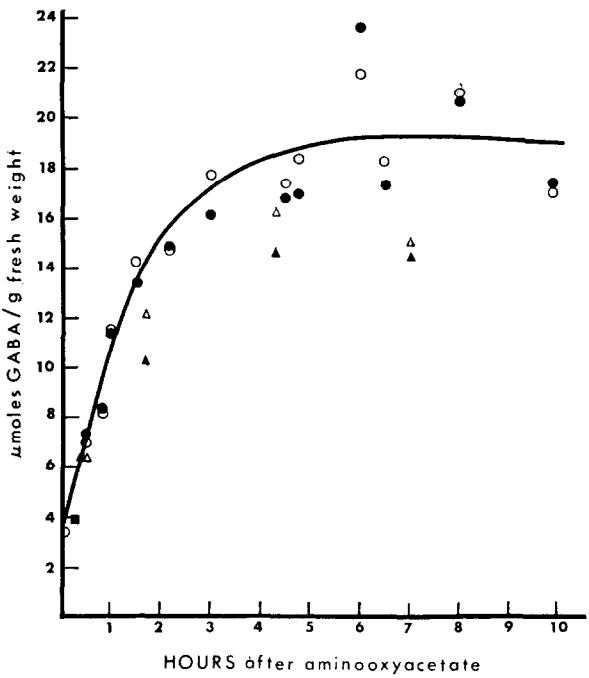

FIG. 4. Effect of aminooxyacetic acid on GABA content in mouse brain. The animals were treated as follows:

○, 40 mg aminooxyacetic acid/kg s.c.; ●, same plus i.p. injection of 500 mg GABA/kg, 3 min to 6 hr after aminooxyacetic acid; △, 20 mg aminooxyacetic acid/kg; ▲, same plus GABA as above; ■, 500 mg GABA/kg i.p.

Each symbol represents an average value from five or more brains.

To complement the histochemical studies, the effect of aminooxyacetic acid on brain GABA levels in mice was determined. The results presented in Fig. 4 show that after a s.c. injection of 20 mg inhibitor/kg, brain GABA levels rose from 3·5 μmoles/g to approximately 15 μmoles/g over a period of 4–5 hr (open triangles). As might be expected from the information presented in Fig. 2b, the results did not change much when the dose of aminooxyacetic acid was doubled to 40 mg/kg (open circles). Both the initial rate and the time course of GABA accumulation remained almost the same, but the final GABA concentration reached somewhat higher values. Similar results were obtained when GABA levels were determined in brain stem instead of in whole brain. The only difference was that values were approximately 20 per cent higher, largely because the dissection required more time.[5]

One aim of the present study was to determine whether GABA-transaminase activity normally prevents GABA in the blood from penetrating into the central nervous system as previous studies had suggested.[4] For this purpose aminooxyacetic acid was administered to mice as usual, after which the animals were injected with 500 mg GABA/kg i.p. (black symbols, Fig. 4). All control animals received saline instead of GABA. As seen in Fig. 4, the values obtained from GABA-injected animals were almost identical with those obtained from mice injected with saline. GABA was administered as early as 10 min after injection of aminooxyacetic acid and as late as 6 hr after the inhibitor. Other animals received GABA at any time between these extremes. This procedure was adopted in order to determine whether the time of GABA injection after aminooxyacetic acid was critical. It also increased the possibility that, at least in the first hour after aminooxyacetic acid, the GABA concentration in blood was higher than that in brain. After i.p. injection of 500 mg GABA/kg, blood concentrations have been shown to reach between 10 and 15 μmoles/ml.[6]

DISCUSSION

From the results presented in Fig. 2 it is clear that aminooxyacetic acid is a strong inhibitor *in vivo* of GABA–α–ketoglutarate transaminase, and that such inhibition is not easily reversed by GABA. Four hours after the administration of 20 mg inhibitor/kg, its concentration in brain sections must have been negligible in comparison with the high GABA concentration (5 mg/ml) in the incubation medium. Aminooxyacetic acid probably inhibits transaminase activity by complexing with pyridoxal phosphate.[7]

The present studies also showed that succinic semialdehyde dehydrogenase was not affected by the doses of aminoxyacetic acid used. Furthermore, the fact that much more rapid formazan production was observed when succinic semialdehyde was used as substrate instead of GABA would tend to suggest that *in vivo* the metabolism of GABA is limited by the rate at which it can be transminated. Although succinic semicaldehyde is a rather unstable compound, appropriate control studies with heated sections (100°) showed that the difference in the rate of formazan production was not an artifact, since little formazan formation occurred in such sections when they were incubated for the same length of time. In heated sections all enzyme activity is presumably abolished.[2]

The curve shown in Fig. 4 indicates that GABA accumulation remained almost constant when the dose of aminooxyacetic acid was doubled from 20 mg/kg to 40 mg/kg. Together with the histochemical evidence presented in Fig. 2, these results suggest that the higher dose was more than adequate to provide maximal inhibition of GABA transaminase. Provided no residual transaminase activity remains which is unaffected by aminooxyacetic acid, it may be assumed that the GABA accumulation curve shown in Fig. 4 reflects the maximal rate *in vivo* of GABA synthesis, under conditions where glucose and glutamate concentrations are presumably normal. At 5–6 μmoles GABA/g per hour, this rate agrees well with that found previously by Elliott and van Gelder[8] in brain slices. Their results showed that in the presence of an estimated concentration of 20–30 μmoles glutamate/g, brain slices can synthesize GABA at a rate of 4–5 μmoles/g per hour. There appear to be other similarities between the present finding and those obtained with slices.[8] Brain slices will absorb GABA rapidly until a maximal concentration of approximately 20 μmoles/g is

reached, and no further absorption occurs thereafter. Similarly, after maximal inhibition of GABA transaminase by aminooxyacetic acid, brain GABA levels *in vivo* increase also until a concentration of approximately 20 μmoles/g is reached. Preliminary experiments with 60 mg inhibitor/kg seem to indicate that GABA levels *in vivo* will not increase much beyond this value.

It might be interesting to determine whether neurons fully saturated with GABA and incapable of metabolizing it would respond for a longer duration to the application of GABA. Absorption of GABA into neurons has been suggested as one method by which the physiological action of GABA is abolished.[9] Alternatively, if the same binding sites for GABA are functioning as physiological receptors, such saturated neurons may not respond any longer to applications of GABA.

The question whether exogenous GABA penetrates into the central nervous system after inhibition of GABA transaminase has not been fully answered by the present work. Although Fig. 4 shows that brain GABA levels in animals injected with both GABA and aminooxyacetic acid did not differ from those found in animals injected with inhibitor alone, these results are not conclusive. First, Elliott and van Gelder[8] showed that the absorption of even small amounts of exogenous GABA by slices appeared to abolish GABA synthesis. This was found even when the final GABA concentration in the slices was still well below the maximal level possible. If the same phenomenon occurs *in vivo*, a possible increase in brain GABA levels by injections of GABA may have been offset by a comparable decrease in endogenous GABA synthesis. This could only be true, however, if the rate of entry of exogenous GABA was exactly matched by a decrease in synthesis, since otherwise the two curves could not have been identical. Another consideration is that mice were used in this study, whereas the most pronounced effect of exogenous GABA was observed in rabbits. It should be remembered, however, that in all species that have been tested, the same resistance toward penetration of GABA into the brain can be demonstrated.[6] It is logical to assume therefore that the mechanism which prevents a net increase in brain GABA levels after its injection into the circulation is the same for all species. Finally, the possibility cannot be discarded that the concentrations of GABA used to test the barrier may have been too low to provide for an adequate concentration gradient from the blood into the brain. This argument may still be valid in spite of the fact that some of the animals were injected with GABA soon after aminooxyacetic acid administration, when endogenous GABA levels had not yet increased very much (Fig. 4). These points will be investigated further.

Despite these objections, however, the evidence at present suggests that inhibition of GABA transaminase in no way facilitates the entry of exogenous GABA into the central nervous system. The observed pharmacological action of GABA after administration of aminooxyacetic acid therefore remains unexplained at this time. Scholes[10] recently reported very similar effects of systemic GABA in young chicks. A peripheral action seems to be ruled out, since injections of GABA alone in many adult species from man to mice (see for example Ref. 6) have never shown the effects described above. If GABA does exert a peripheral action after aminooxyacetic acid, it can only be assumed that the effect is normally not observed, because the peripheral sites at which GABA presumably could act destroy it very rapidly. The existence of such receptors would be a rather startling new concept for which there is at present no evidence.

Acknowledgements—I wish to thank Miss Ann Larratt for her valuable assistance. This investigation was supported by Public Health Service Research Grant NB-04433, from the National Institute of Neurological Diseases and Blindness.

REFERENCES

1. D. P. WALLACH, *Biochem. Pharmac.* **5**, 323 (1961).
2. N. M. VAN GELDER, *J. Neurochem.* **12**, 231 (1965).
3. W. B. JAKOBY and E. M. SCOTT, *J. biol. Chem.* **234**, 937 (1959).
4. N. M. VAN GELDER, *J. Neurochem.* **12**, 239 (1965).
5. K. A. C. ELLIOTT, R. T. KHAN, F. BILIDEAU and R. A. LOVELL, *Canad. J. Biochem.* **43**, 407 (1965)
6. N. M. VAN GELDER and K. A. C. ELLIOTT, *J. Neurochem.* **3**, 139 (1958).
7. B. HABER, *Canad. J. Biochem.* **43**, 865 (1965).
8. K. A. C. ELLIOTT and N. M. VAN GELDER, *J. Neurochem.* **3**, 28 (1958).
9. S. W. KUFFLER and C. EDWARDS, *J. Neurophysiol.* **21**, 589 (1958).
10. N. W. SCHOLES, *Life Sci.* **4**, 1945 (1965).