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A COMPARISON OF γ-AMINOBUTYRIC ACID AND THE SEMI-RIGID ANALOGUES 4-AMINOTETROLIC ACID, 4-AMINOCROTONIC ACID AND IMIDAZOLE-4-ACETIC ACID ON THE ISOLATED SUPERIOR CERVICAL GANGLION OF THE RAT

N.G. BOWERY[1]

Department of Pharmacology, The School of Pharmacy, University of London, 29/39 Brunswick Square, London WC1N 1AX

G.P. JONES

William Ramsay, Ralph Forster and Christopher Ingold Laboratories, University College London, Gower Street, London WC1E 6BT

1 The rat superior cervical ganglion possesses receptors for γ-aminobutyric acid (GABA). This can be demonstrated *in vitro* by recording the changes in ganglionic surface potential which occur after the addition of GABA to the bathing solution.

2 The actions of three conformationally-restricted analogues of GABA namely 4-aminotetrolic acid (4-ATA), *trans* 4-aminocrotonic acid (4-ACA) and imidazole-4-acetic acid (IAA) have been examined for activity at this peripheral receptor.

3 All three analogues depolarized the ganglion in a manner similar to GABA. Their actions were transient and were 'occluded' by GABA; also the log dose-response curve in each case was parallel to that of GABA. Molar potencies relative to GABA (=1) were 4-ACA=1.48, IAA=0.100, 4-ATA=0.0028.

4 The action of each analogue could be blocked by the GABA antagonists bicuculline and tetramethylenedisulphotetramine at doses which had relatively little effect on responses to the cholinomimetic carbachol.

5 4-ACA and IAA (1 mM) significantly reduced the ganglionic accumulation of [^{3}H]-GABA (0.2 μM) by 88% and 58% respectively whereas 4-ATA (1 mM), caused no significant reduction in [^{3}H]-GABA accumulation.

Introduction

4-Aminotetrolic acid (4-ATA), *trans*-4-aminocrotonic acid (4-ACA) and imidazole-4-acetic acid (IAA) (Figure 1) can be considered as conformationally-restricted analogues of γ-aminobutyric acid (GABA) the putative central inhibitory transmitter (see Curtis & Johnston, 1974). All three analogues show considerable GABA-like activity on neurones in the mammalian central nervous system: iontophoretic application produces a marked inhibition in neuronal cell firing, and this can be antagonized by bicuculline, N-methyl bicuculline or picrotoxin. Furthermore they are resistant to the action of strychnine, suggesting that their effect resembles that of GABA rather than glycine (Krnjević & Phillis, 1963; Phillis, Tebecis & York, 1968; Beart, Curtis & Johnston, 1971; Curtis, Duggan, Felix & Johnston, 1971; Curtis, Duggan, Felix, Johnston & McLennan, 1971; Godfraind, Krnjević, Maretić & Pumain, 1973; Haas, Anderson & Hösli, 1973; Dray, 1975; Johnston, Curtis, Beart, Game, McCulloch & Twitchin, 1975).

Receptors for GABA in the mammal are not confined to the central nervous system: depolarization of the cat superior cervical, vagal sensory and dorsal root ganglia *in vivo* (de Groat, 1970, 1972; de Groat, Lalley & Saum, 1972) and the rat superior cervical ganglion *in vitro* (Bowery & Brown, 1974), and release of catecholamines from isolated bovine adrenals (Sangiah, Borowitz & Yim, 1974) can be evoked by GABA. The chemical specificity of the receptors for GABA in the rat superior cervical ganglion (Bowery & Brown, 1974) together with the evidence that GABA depolarizes the ganglionic neurones by

[1] Present address: Department of Pharmacology, St. Thomas's Hospital Medical School, London SE1 7EH.

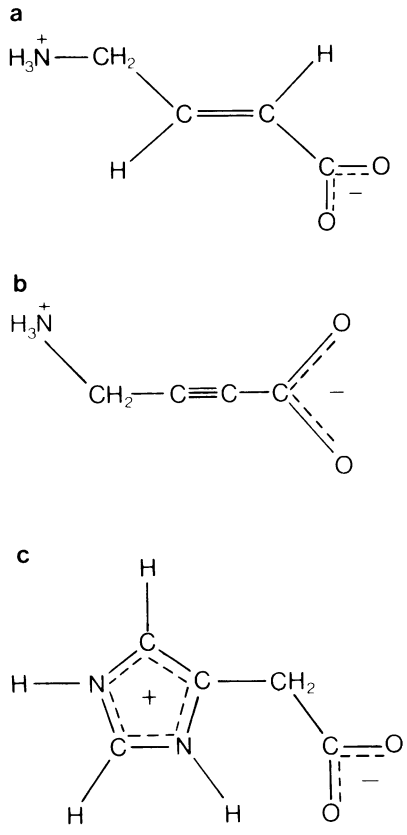

Figure 1 Structures of (a) 4-aminocrotonic acid; (b) 4-aminotetrolic acid and (c) imidazole-4-acetic acid.

increasing Cl^- conductance, (Adams & Brown, 1975) indicates a close resemblance between GABA receptors on sympathetic ganglion cells and those on central neurones. These receptors have certain advantages for experimental study, for instance, drugs can be applied in known concentrations which facilitates potency measurements. In the present experiments we have compared quantitatively the effects of 4-ATA, 4-ACA, IAA and GABA on the rat superior cervical ganglion the results of which may prove helpful in interpreting structural requirements for activation of the GABA receptor.

Methods

Surface depolarization of rat isolated superior cervical ganglia was measured by the superfusion technique described elsewhere (Bowery, Brown & Collins, 1975; Brown & Marsh, 1975). Ganglia were dissected from Wistar rats anaesthetized with urethane (1.5 g/kg) and were desheathed before use.

The effect of the analogues on GABA accumulation by isolated ganglia was determined by incubating ganglia in Krebs solution (1 ml/ganglion) containing 0.2 μM [^{3}H]-GABA ([^{3}H-2-3]-γ-aminobutyric acid, 10 Ci/mmol, New England Nuclear) in the absence or presence of a 1 mM concentration of the analogues. Incubations were continued for 30 min at 25°C in the presence of 10 μM amino-oxyacetic acid to prevent GABA metabolism (Walsh, Bowery, Brown & Clark, 1974). The amount of [^{3}H]-GABA accumulated by individual ganglia was determined by scintillation counting (see Bowery & Brown, 1972a) and expressed as a tissue : medium ratio (d min^{-1} mg^{-1} ganglion : d min^{-1} μl^{-1} bath fluid).

Imidazoleacetic acid was obtained from Sigma Ltd. 4-Aminotetrolic acid and *trans*-4-aminocrotonic acid were prepared by the methods of Beart & Johnston (1972) and Musashi (1954) respectively and subsequently re-crystallized.

(+)-Bicuculline methochloride (methylbicuculline) and tetramethylenedisulphotetramine (TETS) were synthesized by J.F. Collins (Dept. of Chemistry, Sir John Cass School of Science) according to the methods of Johnston, Beart, Curtis, Game, McCulloch & MacLachlan (1972) and Hecht & Heneka (1949).

Results

Depolarization

Superfusion of the rat isolated superior cervical ganglion with solutions containing IAA, 4-ATA or 4-ACA produced a dose-dependent depolarization of the ganglion (Figure 2). The threshold concentration in each case was 5–20 μM (IAA), 300–1000 μM (4-ATA) and 1–3 μM (4-ACA). The depolarizations produced by these compounds showed the following features characteristic of those produced by GABA (Figures 3 and 4).

Firstly, their effects were transient; the peak depolarization occurred within 30 s and then declined in spite of continued application. This contrasted with the depolarization produced by carbachol which was slower in onset and was sustained for at least 4 min (see Bowery & Brown, 1974). Secondly, the log dose-response curves to IAA, 4-ATA and 4-ACA paralleled that to GABA (Figure 2). However, the maximum response to IAA was always less than that produced by GABA, and a maximum response to 4-ATA was never obtained due to its low potency. Thirdly, during prolonged superfusion with GABA, the addition of IAA, 4-ATA and 4-ACA did not depolarize the ganglion whereas carbachol still produced a response. This 'occlusion' phenomenon in the ganglion has previously been described for other GABA analogues (Bowery & Brown, 1972b, 1974).

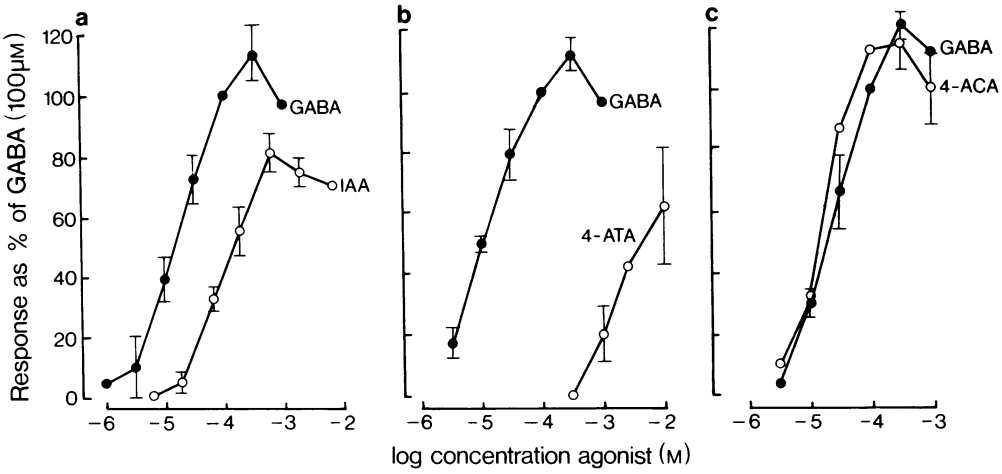

Figure 2 Log dose-response curves for (a) imidazole-4-acetic acid (IAA), (b) 4-aminotetrolic acid (4-ATA), (c) *trans*-4-aminocrotonic acid (4-ACA) and γ-aminobutyric acid (GABA)-induced (●) depolarizations of the rat isolated superior cervical ganglion. Compounds were applied for 1 min at 15 min intervals. Data were obtained from 3 or 4 separate experiments for each compound in which GABA was compared with one or more of the analogues. Each point represents the mean plus or minus standard errors (vertical bars) where $n > 3$. Abscissae: log molar agonist concentration. Ordinates: mV surface depolarization plotted as % of response to GABA 100 μM in the same experiment.

Fourthly, depolarization by the analogues was antagonized by bicuculline or methylbicuculline (Figure 3) and by tetramethylenedisulphotetramine (TETS) (Figure 4) (see Bowery *et al.*, 1975) at concentrations which produced less than 15% reduction of responses to carbachol. It may be noted that the responses to carbachol shown in Figures 3 and 4 were approximately 20% of the maximum response to this substance in these experiments, whereas the responses to GABA of comparable magnitude were approximately 75% of the maximum response to GABA. Moreover at equal levels of depolarization, the

Table 1 The effect of 1 mM concentrations of 4-amino-tetrolic acid (4-ATA), imidazole-4-acetic acid (IAA), *trans*-4-aminocrotonic acid (4-ACA) and γ-aminobutyric acid (GABA) on the uptake of [^{3}H]-GABA by individual ganglia.

	Mean tissue: medium ratio	*% inhibition of [^{3}H]-GABA uptake*	*Depolarization molar potency of analogue*
Control	13.59 ± 0.94 (n=5)		
+ 4-ATA (1 mM)	12.18 ± 1.80 (n=5)	Not significant	0.0028
+ IAA (1 mM)	*5.76 ± 0.59 (n=4)	58%	0.100
+ 4-ACA (1 mM)	*1.58 ± 0.08 (n=5)	88%	1.48
+ GABA (1 mM)	*0.64 ± 0.43 (n=3)	95%	1

*Value significantly different from control ($P < 0.001$).

Ganglia were incubated for 30 min at 25°C in the presence of [^{3}H]-GABA 0.2 μM (plus amino-oxyacetic acid 10 μM). The ganglia were *not* pre-incubated with the analogues. Mean tissue: medium ratios are shown together with the standard error of the mean in each case. The number of ganglia (n) used for each determination is shown in brackets. Molar potencies for depolarization (see text) are included for comparison.

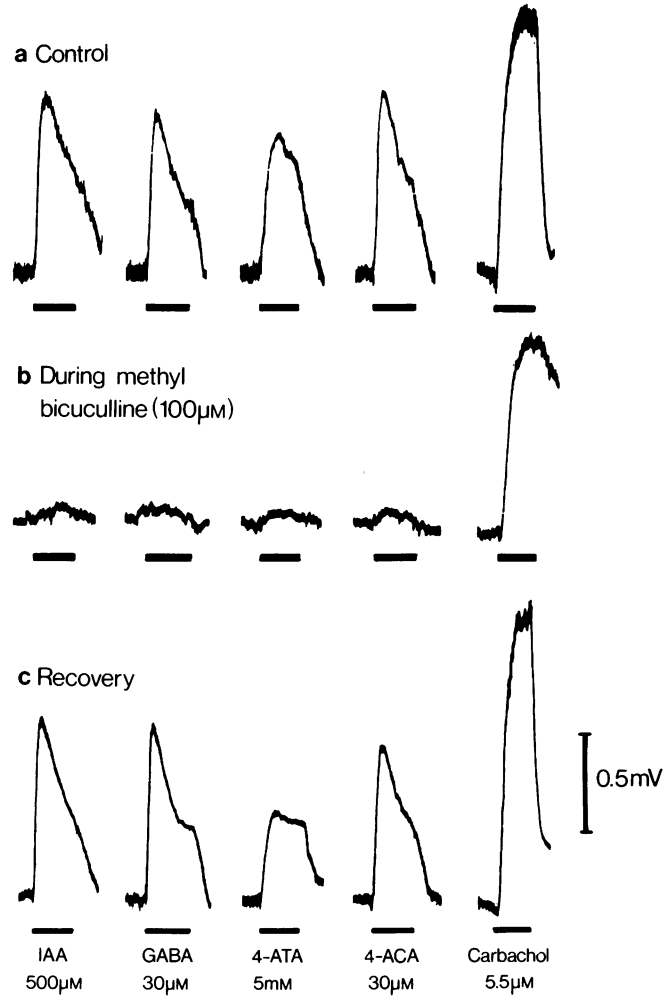

Figure 3 The effect of methyl bicuculline (100 μM) on ganglionic depolarizations produced by imidazole-4-acetic acid (IAA, 500 μM), γ-aminobutyric acid (GABA, 30 μM), 4-aminotetrolic acid (4-ATA, 5 mM), *trans*-4-aminocrotonic acid (4-ACA, 30 μM) and carbachol (5.5 μM). The results were obtained from a single ganglion. Compounds were applied for 1 min (indicated by horizontal bars) at 15 min intervals. Calibration bar 0.5 mV. (a) Was obtained before, (b) during, and (c) 30 min after the application of methyl bicuculline (100 μM). Note the selective antagonism of GABA and its analogues.

conductance change produced by carbachol is much less than that produced by GABA (Adams & Brown, 1975), even though the maximal conductance increase that can be induced by carbachol is far in excess of the maximum induced by GABA. This would tend to make the response to carbachol more sensitive to 'non-specific' reduction than that to GABA.

The molar potencies of the analogues compared to GABA (= 1) determined from the data in Figure 2 by linear regression analysis around the 50% level on the ordinates were IAA = 0.100, 4-ATA = 0.0028 and 4-ACA = 1.48.

Transport of [^{3}H]-γ-aminobutyric acid

Exogenous GABA is accumulated into ganglionic glial cells by a high-affinity carrier-mediated transport process (Bowery & Brown, 1972a; Young, Brown, Kelly & Schon, 1973). Ganglia incubated in low concentrations (< 100 μM) of [^{3}H]-GABA accumulate this amino acid against a concentration gradient. The additional presence of 1 mM IAA or 4-ACA in the incubation medium significantly decreased the uptake of [^{3}H]-GABA during 30 min incubation (Table 1). However, 4-ATA (1 mM) did not significantly reduce

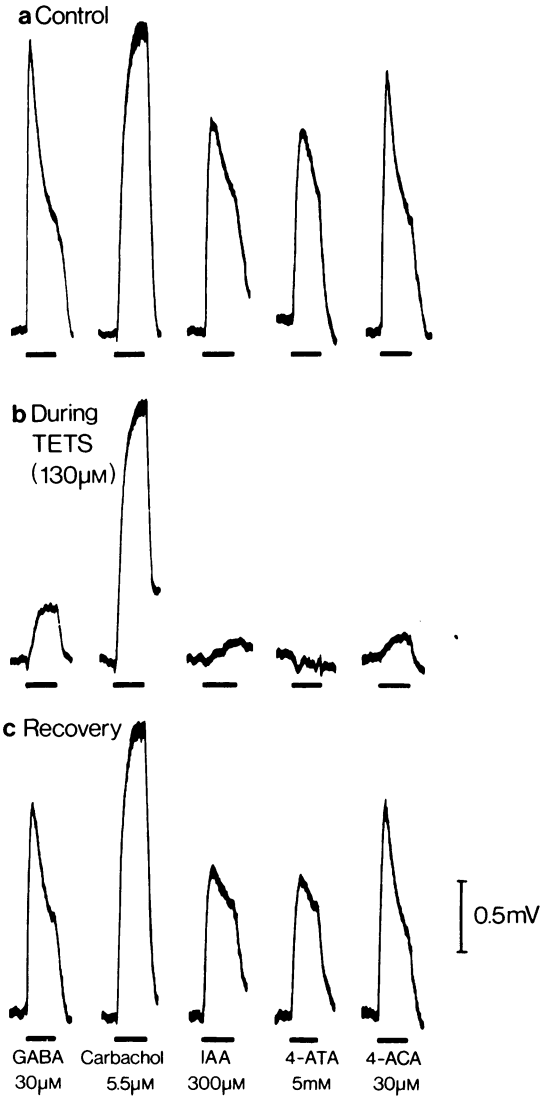

Figure 4 Details as for Figure 3 except that tetramethylenedisulphotetramine (TETS, 130 μM) was used in place of methyl bicuculline and the imidazole-4-acetic acid (IAA) concentration was 300 μM. TETS was dissolved in 0.3% acetone (see Bowery, Brown & Collins, 1975). The results were obtained in a different ganglion from that shown in Figure 3.

GABA accumulation. The order of potency in inhibiting the uptake of GABA was 4-ACA (88%) > IAA (58%) > 4-ATA (10%).

Discussion

The results show that 4-ACA, IAA and 4-ATA all produced a surface depolarization of the isolated superior cervical ganglion in a manner resembling that of GABA. An action at 'GABA receptors' in this tissue (see Bowery & Brown, 1974) is supported in all three cases by (i) the transient nature of responses to the analogues (ii) their parallel log dose-response curves (iii) their 'occlusion' by GABA and (iv) their antagonism by methyl bicuculline and tetramethylenedisulphotetramine. Such an action accords with previous findings in the mammalian central nervous system (see introductory section).

Trans-4-ACA was equipotent with GABA at the ganglionic receptors, a result which compares with that described by Johnston *et al.* (1975). Iontophoretic application of *trans*-4-ACA on to spinal interneurones inhibited cell firing to approximately the same extent as GABA. In contrast, however, the potencies of 4-ATA and IAA in the ganglion (approximately 1/400 and 1/10 of GABA respectively) appear to differ from those estimated at receptors within the central nervous system. Beart *et al.* (1971) indicated that '4-ATA was between one half and one fifth as active as GABA' in depressing spinal interneurones, and other workers have shown that IAA appears to be as active as GABA in decreasing neuronal cell firing in the cerebellum, spinal cord (Curtis *et al.*, 1971) and brain stem (Haas *et al.*, 1973) and about half as active as GABA on cortical neurones (Godfraind *et al.*, 1973). These values are based on electrophoretic currents required to expel sufficient of the drugs to depress cell firing to a similar extent (often maximally); but, even using an alternative means of quantitation (see Hill & Simmonds, 1973) iontophoretically-applied IAA appears to be as potent as GABA in the rat brain stem (Dray, 1975). Nevertheless, a direct comparison between values obtained for IAA and 4-ATA applied iontophoretically in the central nervous system and in the ganglion *in vitro* is difficult in view of the difficulty in expressing potency levels in terms of drug concentration in the former. However, the recent findings of Barker, Nicoll & Padjen (1975) using frog isolated spinal cord indicate that IAA has 2.5 times the potency of GABA in depolarizing primary afferent terminals; and Zukin, Young & Snyder (1974) have shown that IAA is almost as potent as GABA in binding to 'GABA receptors' in synaptic membrane fractions of the rat central nervous system.

Thus, the apparent difference in potencies between IAA and 4-ATA in the central nervous system and ganglion may well be 'real'. Certain factors may contribute to this difference. For example, in brain tissue the accumulation of GABA is greater (cf. Iversen & Neal, 1968 with Bowery & Brown, 1972a) possibly due to the presence of a neuronal uptake component not present in the ganglion (Young *et al.*, 1973). Since both 4-ATA and IAA are poor inhibitors of GABA uptake in cortical or spinal cord slices and brain synaptosomes (Beart, Johnston & Uhr, 1972;

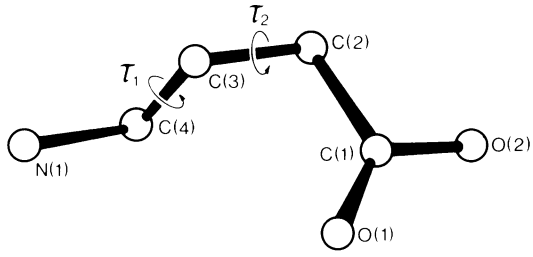

Figure 5 The solid state conformation of γ-aminobutyric acid (GABA) illustrating the torsion angles τ_1 and τ_2 which are used to describe the conformations of the rigid analogues of GABA.

Balcar & Johnston, 1973; Zukin *et al.*, 1974) they might also be accumulated to a much lesser extent thereby increasing their effective concentrations in the region of the receptors.

It is unlikely that the greater potency of IAA or 4-ATA in the central nervous system relates to an additional action at glycine receptors (ganglia are devoid of receptors for glycine, Bowery & Brown, 1974) since strychnine is ineffective as an antagonist of the actions of either 4-ATA or IAA when applied at iontophoretic currents capable of antagonizing glycine (Beart *et al.*, 1971; Curtis *et al.*, 1971; Haas *et al.*, 1973). The insensitivity of IAA to strychnine in the frog spinal cord is confused by the evidence that glycine-evoked primary afferent depolarization is also insensitive to the action of strychnine (Barker *et al.*, 1975).

The molecular conformations of 4-ATA, *trans*-4-ACA and IAA can be illustrated by consideration of the torsion angles (Klyne & Prelog, 1960) τ_1 (N(1)–C(4)–C(3)–C(2)) and τ_2 (C(4)–C(3)–C(2)–C(1)) depicted on the GABA molecule shown in Figure 5. In 4-ACA, τ_2 is fixed at $\sim 180°$ with free rotation occurring about the bond defined by τ_1. 4-ATA is linear because of the stereochemical requirements of the triple bond although free rotation is allowed about C(3)–C(4). IAA has τ_1 fixed at $\sim 180°$. In each of these molecules the separation of the negatively charged centre and the positively charged nitrogen atom of 5.8 ± 0.2 Å suggested by Kier, George & Höltje (1974) for activity at the GABA receptor is theoretically possible. X-ray crystal structure analyses on these compounds (Jones & Pauling, 1975, 1976a,b) show that in the solid state this is in fact the case (see Table 2).

Unlike the primary amine groups of GABA, 4-ATA and 4-ACA, IAA has a secondary amine functionality which is fixed in a planar configuration by the requirements of the imidazole ring, with only one hydrogen atom available for hydrogen bonding. If hydrogen bonding is important in the binding of the amine centre to the receptor then IAA is likely to be less effective than GABA. The fixed planar configuration appears to be important since N-methyl GABA, where the nitrogen atom is allowed to rotate freely about the N(1)–C(4) bond, has negligible activity both in the central nervous system (Curtis & Watkins, 1960) and in the ganglion (Bowery & Brown, 1974).

It has been suggested by Beart *et al.* (1972) that in the case of inhibition of GABA uptake by 4-ATA in rat brain slices the stronger acidity of 4-ATA may account for its weaker inhibition when compared with other structural analogues of GABA. This may also be an important factor when considering binding at the receptor. The low pK_1 (COO^-) value of 4-ATA (1.08) suggests a stronger ionic binding or hydrogen bonding capability of the carboxylate end of the molecule which may lead to an adverse distortion of the receptor conformation. Conversely, the low pK_2 (NH_3^+) value for IAA (7.46) suggests that the nitrogen centre may be less effective than in GABA (pK_2 10.71) for binding to the receptor. 4-ACA has similar pK_1 and pK_2 values to GABA. The differences in the action of 4-ATA and IAA in the central nervous system and in the ganglion may therefore illustrate differences in charge requirements of the substrates for these two systems.

The authors wish to thank Dr J.F. Collins for the preparations of N-methyl bicuculline and tetramethylenedisulphotetramine.

Table 2 Intramolecular N . . . . . O distances (Å) for the restricted γ-aminobutyric acid (GABA) analogues

	N . . . O[1]	*N . . . O[2]*	*Reference*
GABA	5.612 (±.006)	4.238 (±.007)	Tomita, Higashi & Fujiwara (1973)
4-ACA	5.657 (±.003)	5.052 (±.003)	Jones & Pauling (1975)
	5.098 (±.003)	5.130 (±.003)	
4-ATA	5.699 (±.004)	5.227 (±.004)	Jones & Pauling (1976a)
IAA	5.714 (±.004)	4.402 (±.004)	Jones & Pauling (1976b)

Estimated standard deviations in parentheses. 4-ACA = *trans*-4-aminocrotonic acid; 4-ATA = 4-aminotetrolic acid; IAA = imidazole-4-acetic acid.

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