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MICRO-VACUOLATION IN RAT BRAINS AFTER LONG TERM ADMINISTRATION OF GABA-TRANSAMINASE INHIBITORS

COMPARISON OF EFFECTS OF ETHANOLAMINE-O-SULPHATE AND VIGABATRIN

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Abstract—Two "suicide" inhibitors of GABA-aminotransferase which are known to raise the concentration of GABA *in vivo* and to have anti-convulsant properties, have been compared for the extent to which they produce micro-vacuoles in the brains of rats. The compounds γ-vinyl-GABA (Vigabatrin) and ethanolamine-O-sulphate were administered orally for six months to rats at doses that produced the same increase in brain GABA levels. Micro-vacuolation was found to be present in the brains of animals treated with either compound but to be more severe in those treated with Vigabatrin. A quantitative assessment using computerised image analysis revealed that both the number of vacuoles, and the area occupied by them, was twice as high in the Vigabatrin treated animals as in those treated with ethanolamine-O-sulphate. This quantitative difference could be seen to be due to the fact that in the Vigabatrin treated animals the vacuoles extended into the white matter tracts between the cerebellar folia whereas in those animals treated with ethanolamine-O-sulphate it was confined to the roof nucleus.

Current hypotheses on the underlying neurochemical abnormalities in epilepsy suggest that augmentation of the inhibitory mechanisms may prove a useful approach to the design of new drugs for epilepsy [1–3]. The possibility of achieving anticonvulsant effects by raising the concentration of the inhibitory neurotransmitter 4-aminobutyrate (GABA) in brain has stimulated the design of various compounds intended as specific inhibitors of GABA-aminotransferase. Several mechanism-based ("suicide") inhibitors have been synthesised [4–8] because of the expectation that this particular inhibitory mechanism should be accompanied by a high degree of specificity for the target enzyme. Two of these compounds, ethanolamine-O-sulphate (aminoethyl hydrogen sulphate, EOS) and 4-aminohex-5-enoate (γ-vinyl-GABA, Vigabatrin) show the anticipated high specificity *in vitro* [4, 7, 9]. They also achieve extensive inactivation of brain GABA-aminotransferase *in vivo* and produce large rises in brain GABA concentration [10, 11]. Both compounds have been shown to have anti-convulsant effects in various experimental animal models, for example giving protection against audiogenic seizures [12, 13]. Vigabatrin has been tested with considerable success in several clinical trials as an anti-epileptic [14, 15]. EOS has not been tried clinically.

The clinical success of Vigabatrin as an anti-epileptic is marred by one adverse toxicological observation namely that after prolonged treatment with very high doses of this compound some experimental animals (rats, mice, cats and dogs) develop pronounced vacuoles in some tracts of the brain. The question arises as to whether these vacuoles are an inevitable consequence of elevated GABA concentrations or whether they result from an additional unsuspected effect of Vigabatrin. Should micro-vacuolation be caused directly by high brain GABA concentrations then any compound that raises brain GABA levels by inhibiting GABA-aminotransferase would be expected to share the same adverse toxicology. Although EOS is chemically very different from Vigabatrin, when administered orally it also inactivates brain GABA-aminotransferase extensively and maintains high brain GABA concentrations over long periods [10].

In this paper we report the results of experiments intended to determine whether high GABA levels brought about by oral administration of EOS produce vacuoles comparable with those arising from oral administration of Vigabatrin.

EXPERIMENTAL

Chemicals. The method of Phillips and Fowler [15] was used to purify EOS (Koch Light Ltd., U.K.). Vigabatrin was generously donated by Merrell International Research Centre, Strasbourg, France.

Animals. MRC Hooded rats were maintained on

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Heygate modified diet 41B (Pilsbury Ltd., Edgbaston, U.K.) fed *ad libitum*. Animals of the same sex were kept four to a cage and the drugs were administered in the drinking water at a concentration of 3 g l^{-1}. In order to overcome the resistance of the group receiving Vigabatrin to drinking a solution of that compound at that concentration, sucrose (1 g l^{-1}) was also included in the drinking water of all the animals. At the beginning of the periods of administration the animals were aged 10 weeks and weighed 210 ± 20 g (male) and 180 ± 5 g (female). At the end of the study rats were killed by an overdose of ether.

Histology. Brains used for histological analysis were fixed in a solution of ice-cold, neutral, phosphate buffered formaldehyde (10% v/v commercial Formalin in 70 mM potassium in ice-cold water). The hemispheres, brain stem and cerebellum were divided through the sagittal plane and whole sections were cut at 1 mm thickness and processed for embedding in paraffin wax. Sections were cut at 5 μm and stained with haematoxylin and eosin. Some sections were stained with Kluver–Barrera stain for myelin and Nissl substance.

Enzyme and neurotransmitter assays. Brain was processed with a glass Teflon homogeniser to give a 25% w/v homogenate. This was used directly for enzyme assay and 0.5 ml of it was added to 5 ml of methanol at −20° for analysis of GABA. Both groups of test animals and the control group were treated identically and all operations were carried out as quickly as possible.

GABA was assayed by HPLC following pre-column derivatisation with *o*-phthaldialdehyde. Chromatographic separation was achieved on a 5μ C18 Spherisorb column using a mobile phase consisting of water:sodium propionate (0.15M):acetonitrile:methanol:dimethylsulphoxide, 61:13:16:9:1. The mobile phase was adjusted to pH 5.8 with 3 M phosphoric acid. Flow was maintained at 1.5 ml min^{-1} and the column eluate was monitored with a fluorescence detector set at an excitation wavelength of 254 nm and an emission wavelength of 418 nm. Ethanolamine was used as internal standard.

GABA-aminotransferase was measured by adding brain homogenate (0.1 ml, 25% w/v) to 0.4 ml of a solution of substrates in 0.1 M borate pH 8.4. Experimental conditions have been described elsewhere [16].

Quantitation of micro-vacuolation. A computerised method was developed to assess the degree of micro-vacuolation. This used an IBAs II television image analyser (Kontron GmbH, Munich) which allowed monochrome pictures received from the microscopy and associated television camera to be stored, manipulated and enhanced. The brain sections were observed under a ×4 objective, the roof nucleus area being positioned in the centre of the field. The television image was stored as 512 × 512 picture elements (pixels). Each element was assigned a "greyness" value varying digitally between 0 (black) and 255 (white). The system was capable of making a clear distinction between vacuoles and other areas except that some portions of the section present in both test and control brains gave the same low "greyness" score as the vacuoles. These areas, much larger than the vacuoles and clearly recognisable, were eliminated from the analysis using an operator controlled digitizer tablet.

RESULTS

No immediately obvious and consistent difference in either appearance or behaviour was observed between the control groups and animals receiving either EOS or Vigabatrin. In particular no convulsions were seen in any of the animals. However, the possibility of occasional convulsions cannot be excluded because observation was not continuous. It was clear that the animals receiving Vigabatrin drank less than the control or EOS groups which were the same in this respect. Over the full period of the investigation consumption of water by the groups receiving Vigabatrin was 80% that of the control and EOS groups. The average dose received by rats administered Vigabatrin was 312 mg kg^{-1} day^{-1} and that received by the group treated with EOS was 366 mg kg^{-1} day^{-1}. Rats receiving Vigabatrin gained less weight than the control groups or those receiving EOS. The mean weights of male rats in control, EOS and Vigabatrin groups were (±SD) 401 ± 7 g, 409 ± 39 g, and 327 ± 21 g respectively. Weights of female rats presented in the same order were 247 ± 14, 243 ± 14 and 210 ± 17.

Effects of the inhibitors on brain GABA aminotransferase and on brain GABA

During the present comparative study of the histological effects of these two compounds in some animals treated with EOS we have analysed enzyme and neurotransmitter levels in one half of the brain while using the other half for histological investigation. The results, presented in Table 1, confirm that long term oral dosage with this compound produces very similar biochemical effects to those produced by Vigabatrin. The enzyme is inactivated by about 75% and GABA is raised approximately two-fold. Qualitative histological examination of the brains from EOS treated rats suggested that vacuolation if present was very slight but at this point a direct histological comparison with Vigabatrin treated rats was not made.

Histological comparison

In order to make a direct comparison of the histological effects of the two compounds groups of eight rats (four of each sex) aged 11 weeks were treated for 6 months either with EOS or with Vigabatrin at 3 g l^{-1} in drinking water. A control group of the same age, sex and number was maintained under the same conditions but not treated with either compound. One animal from the EOS group developed a superficial abdominal tumour and was removed from the study. Stained sections of the brain of all rats were examined blind. The striking abnormality in the drug-treated animals was the presence of microvacuoles in the white matter being most prominent in the region of the roof nuclei and white matter between the cerebellar folia. The neurones appeared normal and there was no glial reaction to the presence of vacuole formation. These vacuoles were much more marked in the Vigabatrin rats than

Table 1. Levels of GABA-aminotransferase and GABA in rats after long term administration of EOS and Vigabatrin

Period of administration	Number of animals	GABA-aminotransferase (μmol min^{-1} g^{-1} tissue)	GABA (μmol g^{-1} tissue)
6 months EOS	2	0.35 ± 0.01	4.92 ± 0.54
9 months EOS	12	0.23 ± 0.01	5.84 ± 0.74
3 months Vigabatrin	2	0.28 ± 0.03	4.10 ± 0.58
6 months Vigabatrin	3	0.30 ± 0.03	6.23 ± 0.3
Control	12	1.18 ± 0.18	2.76 ± 0.19

Figures are expressed ± SD except where N = 2 in which case the results are presented together with ranges.

Table 2. Quantification of micro-vacuolation

Groups	Mean number of vacuoles per field	Mean area of field occupied by vacuoles	Median size of vacuoles (μm)
Controls	8.0	0.07	109
(N = 8)	(2.6 to 24.6)	(0.02 to 0.29)	(74 to 162)
EOS	86.43*	0.75*	122
(N = 7)	(35 to 211)	(0.32 to 1.78)	(108 to 138)
Vigabatrin	172.77*	1.503*	141†
(N = 8)	(78 to 382)	(0.56 to 4.03)	(110 to 182)

Each parameter is presented as the geometric mean for each group of rats. The figures in brackets denote values between which 95% of the population values are estimated to lie. All measures were obtained in duplicate for each brain section. Significant difference from control group * $P < 0.001$, † $P < 0.01$. Significant difference from EOS group $P < 0.02$.

in those treated with EOS. In particular in the Vigabatrin-treated rats the vacuoles extend prominently into the white matter tracts of the cerebellum. No differences in the extent of vacuolation in brains of males and females was evident. Figure 1 shows photomicrographs of the cerebellar region of the brains of all animals.

Quantification of the micro-vacuolation

Previous quantification of the micro-vacuolation caused by Vigabatrin has rated the degree of severity of the lesion subjectively on a scale from 0 to 5 (Centre de Recherche Merrell, internal report). In the present study, where it was clear by eye that both groups of test rats suffered the lesions to some extent with the Vigabatrin group being more severely affected, we wished to assess the severity on a more soundly based quantitative basis. The results (Table 2) obtained using the image analyser serve to reinforce the subjective conclusions already drawn. Using the Kruskal–Wallis one way analysis of variance the Vigabatrin-treated rats had significantly more vacuoles ($P = <0.02$) and the median size of the vacuoles was greater ($P \leq 0.02$) than the EOS-treated rats.

DISCUSSION

Our observation that the long term oral administration of either EOS or Vigabatrin to rats produces similar extents of inactivation of brain GABA-aminotransferase as well as similar increases in brain GABA concentrations confirms the observations of others made using these compounds separately [10, 11, 14, 17]. We would expect therefore that any toxic effects arising directly from long term persistence of high brain GABA concentrations would appear equally in animals receiving either compound. Examination of the cerebellar sections taken from these animals receiving Vigabatrin shows very clear and extensive micro-vacuolation. As far as we know this observation has not previously been published although the observation of these vacuoles during toxicological screening has been the subject of published correspondence [18]. The effective therapeutic dose with which human epileptic patients are treated is about 50 mg/kg/day, i.e. about one-sixth of the dose that produces vacuoles in rats as shown in Fig. 1. Although the degree of micro-vacuolation produced by EOS is much less severe than that produced by Vigabatrin and does not extend into the white matter tracts, some vacuoles, more numerous than in the controls, are present in the roof nucleus.

The cause of the microvacuoles remains mysterious. The presence of vacuoles in the EOS treated rats, however limited, makes it impossible to rule out elevated GABA as the direct cause. Nevertheless, bearing in mind that the levels to which GABA is raised by either compound are remarkably similar, it is worth considering that the presence of the compounds themselves, both of which are GABA-analogues and both of which have been administered at high doses, has some direct effect that results in micro-vacuolation not mediated by high GABA concentrations. Sufficient data are available concerning the kinetic parameters governing the inactivation *in vitro* to make a rough estimate of the concentration of Vigabatrin to which the enzyme must be subjected in order to achieve the observed rates of inactivation *in vivo* with half time of approximately one hour [7]. This would require a concentration of Vigabatrin of

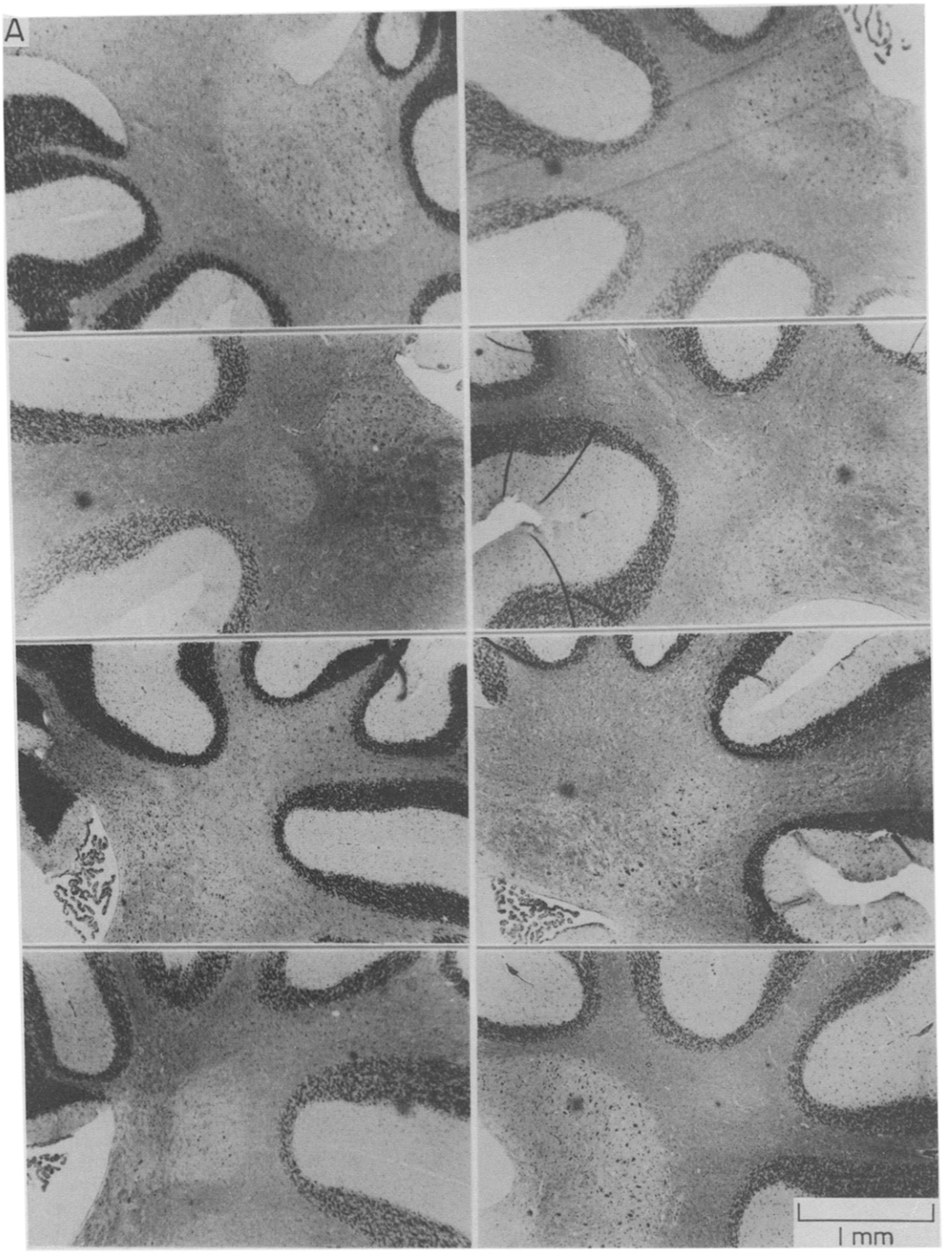
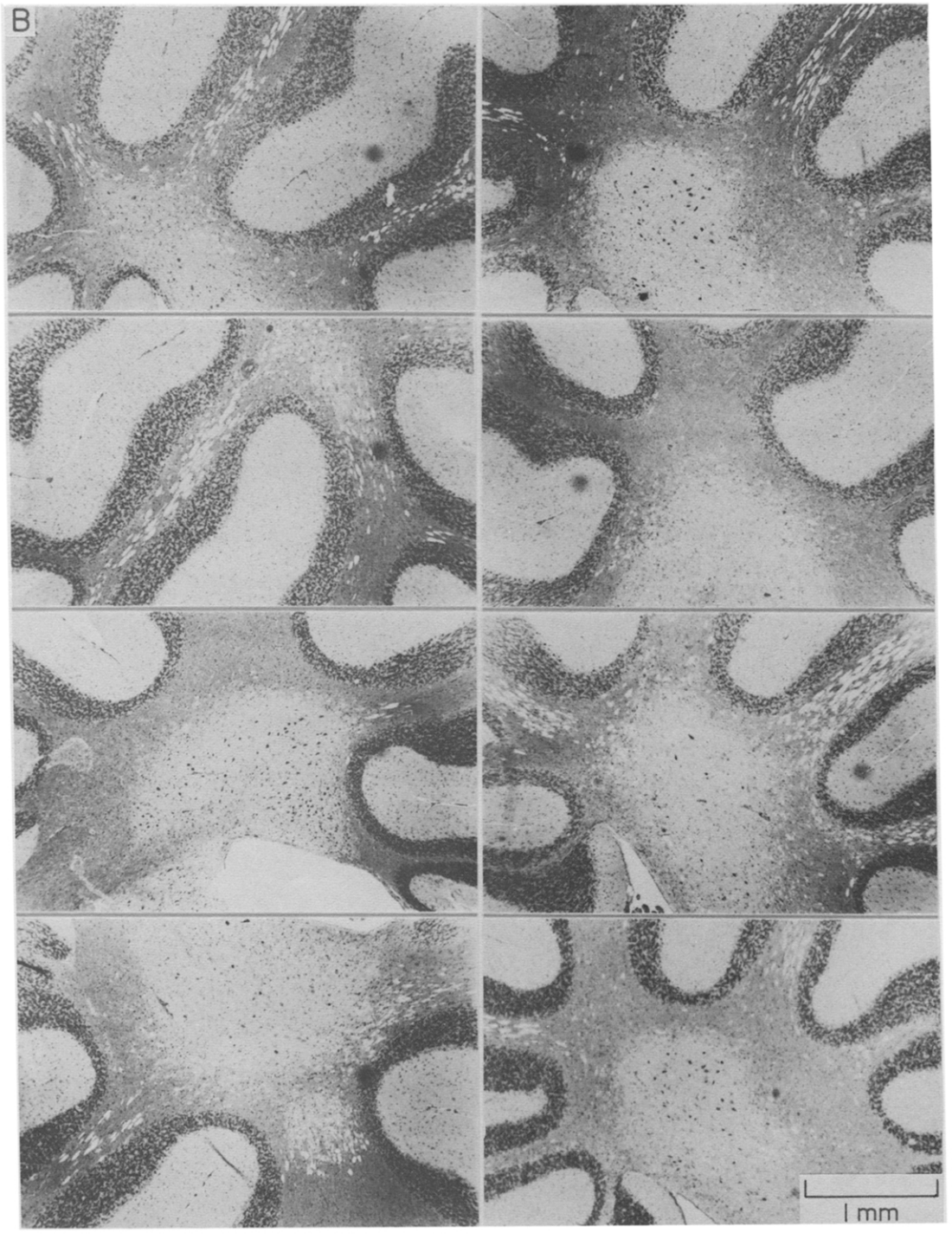


Fig. 1 (*continued*)



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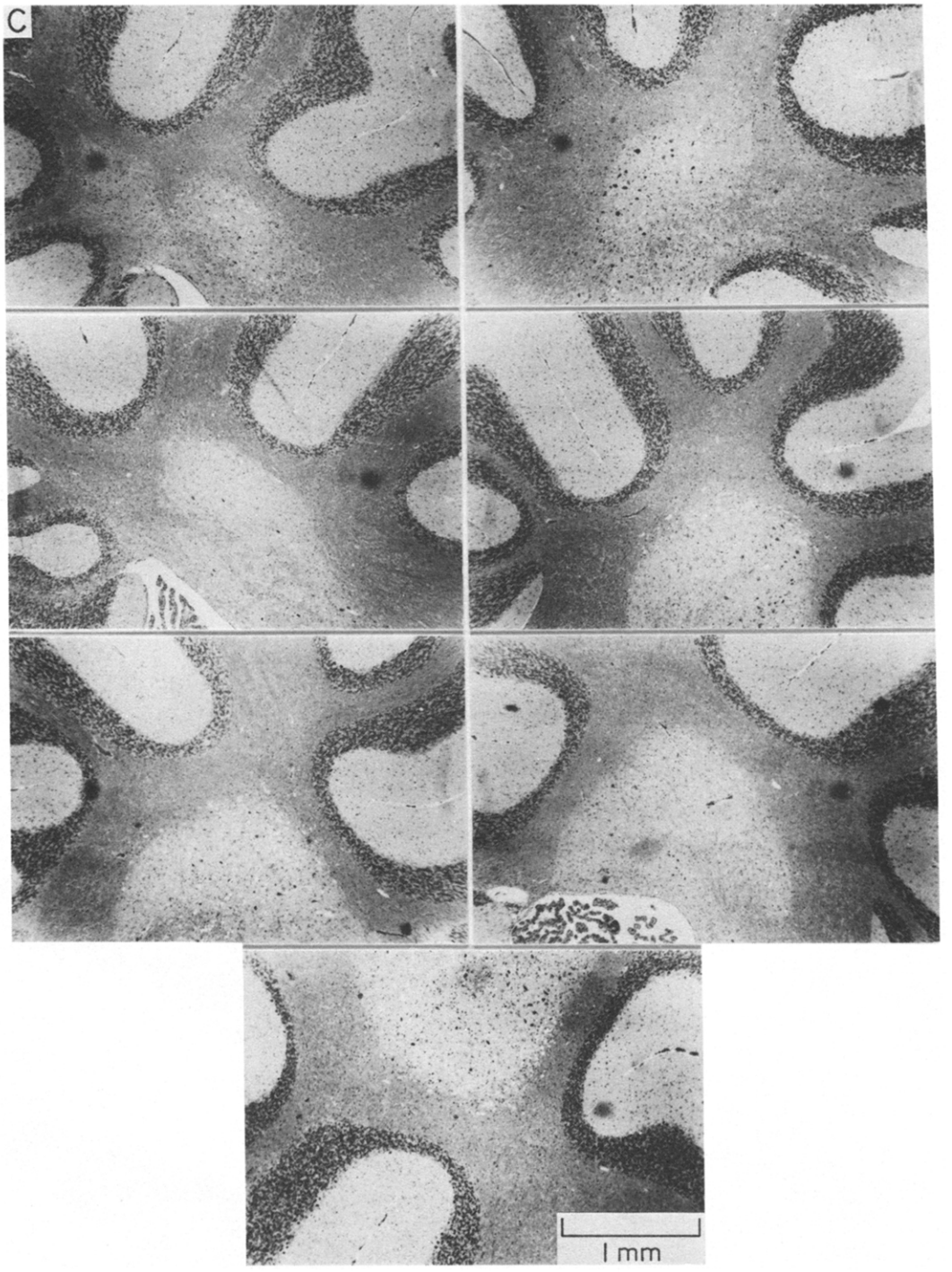

Fig. 1. Photomicrographs of the cerebrellar region of (A) control rats, (B) those treated with Vigabatrin and (C) those treated with EOS. The images are photographs of the specimens from which the data of Table 2 were obtained.

about 0.4 mM. It seems possible that the maintenance of concentrations of the compound of this order over such long periods may be the cause of the problem. Another possibility that we have considered is that, although Vigabatrin is very selective in inactivating GABA-T and does not in the process of inactivating this enzyme release any product into the medium it is a substrate for at least one other aminotransferase. Although ornithine aminotransferase is not inactivated by Vigabatrin examination of the reaction of the compound with this enzyme by absorption spectrum analysis [9] showed that transamination took place. The product of the reaction is presumably the ketone analogue of Vigabatrin a compound whose expected reactivity might be problematical.

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