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Measuring changes in cerebral glutamate and GABA metabolism prior to convulsions induced by 3-mercaptopropionate

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It is well established that, by manipulating the GABA system in the brain, it is possible to alter the convulsive threshold [1]. Several convulsant drugs have been shown to inhibit glutamate decarboxylase (L-glutamate 1-carboxylase, EC 4.1.1.15, GAD) and, in some cases, reduce the brain GABA level. Compounds of this type include methoxypyridoxine [2], mercaptopropionic acid [3], thiosemicarbazide [4], allylglycine [5], hydrazides [6] and mercapto amino acids [7]. On the other hand, inhibitors of GABA aminotransferase (EC 2.6.1.19, GABA-T) have been shown to be anticonvulsant; such compounds include amino-oxyacetic acid [8], ethanolamine *O*-sulphate [9], cycloserine [10] and di-*n*-propylacetate [11], which has subsequently proved useful as an anti-epileptic (Epilem®, Depikine®), [12].

On the basis of the evidence obtained using these and other compounds, various attempts have been made to directly correlate the degree of GAD inhibition and brain GABA level with the convulsive threshold [13–15], but this has proved difficult for two main reasons. First, several of the compounds listed are carbonyl-trapping agents which will react with all pyridoxal phosphate dependent enzyme systems although GAD is usually the most sensitive to inhibition by this means [7, 16]. In addition, the specificity of action of allylglycine [17] and di-*n*-propylacetate [18] have recently been questioned. The second problem concerns the measurement of changes occurring in glutamate and GABA metabolism *in vivo*. The *in vitro* assay of GAD or GABA-T activity in a brain homogenate will not indicate accurately the level of inhibition *in vivo* in the presence of a reversible inhibitor such as mercaptopropionate [19], since the inhibitor will be diluted from its *in vivo* concentration. Also, the measurement of overall brain GABA levels will not necessarily detect changes in GABA synthesis within specific inhibitory nerve terminals if, as is thought [4], GABA metabolism is compartmented in a similar way to glutamate.

The first of these problems can be overcome by the development of new specific active site directed inhibitors of the pyridoxal dependent enzymes [20, 21], and the aim of the present paper is to indicate possible methods for overcoming the neurochemical assay problems. A preliminary account of some of this work has already been reported [22].

MATERIALS AND METHODS

Adult LACG mice of either sex weighing 30–35 g were used in all the experiments. Radiochemicals were obtained from the Radiochemical Centre, Amersham, England. Other reagents were obtained from either the Sigma Chemical Co., London, or the Boehringer Corporation (London) Ltd.

Mice were injected intraperitoneally (i.p.) with either mercaptopropionate (MP, 1.4 m-moles/kg body wt) in the form of the sodium salt or saline (0.9%, w/v) as control. 1 min prior to the intracerebroventricular injection of 0.05 μmoles of [U-^{14}C]pyruvate (10 μCi/mole) or 1 μmole of L-[U-^{14}C]glutamate (0.5 μCi/μmole) in 5 μl of saline. Controls received 5 μl of saline alone. After a further 3 min mice were killed either by cervical fracture and exsanguination (for subsequent assay of GAD activity) or by total immersion in liquid N_2 (for the subsequent assay of amino acid levels and determination of the incorporation of ^{14}C into GABA and glutamate).

For amino acid assays the whole brain was dissected out whilst still frozen, weighed, and homogenized in 94% ethanol (v/v) at 1 ml/200 mg of tissue. After standing for 20 min at 0° the homogenate was centrifuged at 800 *g* for 10 min. An aliquot (20 μl) was taken for scintillation counting and a similar aliquot applied to the midline of a 30 × 50 cm Whatman No. 1 paper and electrophoresed at pH 4 for 20 min at 4000 V. Spots were detected by spraying with ninhydrin and identified by means of standard amino acid samples run simultaneously on each paper. Spots corresponding to GABA, glutamate, aspartate and the neutral material remaining at the origin were cut out and counted. To ensure that the activity measured in the individual amino acid spots was homogeneous and not partially due to contamination, some spots were cut out, pooled, eluted overnight in 0.5 ml of 50% (v/v) ethanol, evaporated down to 0.05 ml and subjected to single dimensional silica gel TLC in a butanol:propionic acid:water (9.2:4.7:6.1) system. Subsequent counting of 0.5 cm strips of the plate indicated that all the radioactivity occurred in the area corresponded to the appropriate amino acid. Correction factors were calculated for the recovery of amino acids from the electrophoresis paper and also for quenching and the efficiency of the scintillation counter. Results were calculated as dis/min/mg of brain tissue. In some samples the total amino acid levels were determined by the dansylation of 10 μl aliquots of the ethanol supernatant by the method of Roberts and Keen [23] as modified by Taberner and Keen [24].

The extraction of brain tissue and assay of GAD activity were carried out essentially as described earlier [19]. The final concentrations in the assay medium (2.0 ml) were as follows:—brain tissue: 0.25 mg/ml; L-glutamate (0.5 μCi): 10 mM; pyridoxal phosphate: 0.3 mM; sodium phosphate buffer (pH 6.4): 50 mM. In the assays performed under limiting substrate concentrations no cold exogenous glutamate was added so that the final concentration of added L-glutamate was 0.011 mM (23 μCi/μmole). In these assays a portion of the final incubation medium was taken and assayed for the glutamate derived from the endogenous tissue concentration. By this means the initial specific activity of the glutamate in the incubation medium was calculated.

Calculation of theoretical enzyme inhibition. The initial assumption was made that the kinetics of GAD conformed to the Michaelis–Menten model so that, in the presence of MP, a reversible competitive inhibitor with respect to glutamate, the following relationship will hold:

$$\frac{v}{v_i} = 1 + \frac{K_m}{K_i}\left|\frac{i}{K_m + s}\right|$$

Table 1. Inhibition of glutamate decarboxylase by 3-mercaptopropionate

Assay Conditions	Theoretical Inhibition	Observed Inhibition
In Vitro Mercaptopropionate: 0.35 mM Glutamate: 25 mM (excess substrate added)	53.8%	48.1 ± 2.3%
In Vitro Mercaptopropionate: 0.35 mM Glutamate 2.5 ± 0.14 mM (no exogenous glutamate)	*89.6 ± 8.2%	82.7 ± 5.2%
In Vivo Glutamate: 10 mM Mercaptopropionate: 1.4 mM	91.4%	

Mice were injected i.p. with MP and killed after 4 min. For calculation of the theoretical inhibition see Methods. Results of observations are the mean ± S.E.M. of 8 observations.

* Median ± 95 per cent confidence limits.

where v = reaction rate in the absence of MP, v_i = reaction rate in the presence of MP at concentration i (mM), K_m = Michaelis constant for GAD = 1 mM, K_i = the inhibitor constant for MP = 0.012 mM (7), s = concentration of L-glutamate. For the *in vitro* assays the values of i and s are known, but *in vivo* it is necessary to make two further assumptions. First, that the value of s corresponds to the known concentration of glutamate in the appropriate brain region (for the cortex this was found to be 9.9 ± 0.9 mM) and, secondly, that the injected MP is evenly distributed so that the brain concentration can be related to the total dose administered and the body weight of the mouse (i.e. ~ 1.4 mM). The results were calculated so that the inhibited reaction rate was expressed as a percentage of the corresponding control reaction rate.

All the results, where appropriate, have been calculated as the mean ± S.E.M. of at least 6 determinations. Students t test was used to determine the significance of the differences between the means of independent groups.

RESULTS AND DISCUSSION

The theoretical and observed inhibition of GAD by MP is shown in Table 1. It can be seen that the theoretical inhibition calculated for the *in vitro* assay (53.8 per cent) is in close agreement with the value actually measured (48.1 ± 2.4 per cent). Also, the theoretical inhibition *in vivo* (91.4 per cent) is in close agreement with that obtained by omitting exogenous cold L-glutamate from the *in vitro* assay (89.6 per cent). The actual value obtained under these conditions (82.7 ± 5.2 per cent) thus gives a closer estimate of the likely *in vivo* inhibition than does the usual assay performed under excess substrate conditions. This method is only applicable to completely reversible inhibitors since it is only then that the dilution of the inhibitor from its *in vivo* concentration results in an underestimate of the effective inhibition *in vivo*. Previous workers [25] have sought to overcome this problem by increasing the tissue concentration in the assay medium, however this also increases the endogenous substrate concentration as well as the enzyme concentration. In the present method, lower concentrations of tissue may be used since the concentrations of enzyme, substrates and inhibitor are all being diluted to the same extent. However, the final concentration of endogenous substrate in the

Table 2. Relative incorporation of ^{14}C into amino acids from L-[U-^{14}C]glutamate or [U-^{14}C]pyruvate injected intracerebroventricularly

Precursor (given at t = 1 min)	L-[U-^{14}C]glutamate: 1 μmole		[U-^{14}C]pyruvate: 0.05 μmole	
Pretreatment (i.p. injection at t = 0 min)	Saline	1.4 m-moles/kg Mercaptopropionate	Saline	1.4 m-moles/kg Mercaptopropionate
Mice killed at t = 4 min				
Total counts in supernatant (dpm/20 μl)	2159 ± 214 N.S.	2119 ± 221 P > 0.05	887 ± 91 N.S.	1017 ± 68 P > 0.05
% of total counts in:				
Glutamate	48.2 ± 3.6 N.S.	46.8 ± 2.5 P > 0.05	20.7 ± 3.4 N.S.	26.9 ± 2.4 P > 0.05
GABA	0.93 ± 0.21	0.49 ± 0.05 P < 0.05	3.02 ± 0.43	0.79 ± 0.07 P < 0.01
Aspartate	8.3 ± 1.02 N.S.	6.9 ± 0.78 P > 0.05	7.8 ± 0.50 N.S.	7.6 ± 0.42 P > 0.05
Neutral fraction	37.2 ± 2.6 N.S.	40.8 ± 2.9 P > 0.05	10.3 ± 0.91 N.S.	12.1 ± 1.1 P > 0.05
Unaccounted label	5.37	5.01	58.2	52.6

Data represent the means ± S.E.M. from groups of six observations.
P values calculated with respect to the equivalent saline control.

assay should be assayed independently in case the inhibitor alters its endogenous concentration and thus affects the reaction rate indirectly.

The relative incorporation of ^{14}C from L-[U-^{14}C]glutamate and [U-^{14}C]pyruvate into GABA and glutamate is shown in Table 2. Absolute estimates of the rate of GABA turnover obtained by observing the fate of labelled precursors such as [U-^{14}C]glucose or [2-^{14}C]acetate involve several important assumptions due, in part, to the compartmentation of glutamate [26], although the half-life of endogenous GABA is believed to be about 10 min [27]. Alternative approaches consist of determining the relative half-lives of exogenous labelled glutamate and GABA [28], or the relative specific activities of glutamate and GABA [29]. [U-^{14}C]glutamate has been used in the present work as well as [U-^{14}C]pyruvate since the latter has the advantage of yielding a greater proportion of counts in GABA over short periods, thereby reducing the error involved in measuring very low count rates. From Table 2 it can be seen that, with either precursor, the total counts in the supernatant fractions did not vary significantly between saline and MP treated mice, thus indicating that the injection procedure was consistent. Following the injection of L-[U-^{14}C]glutamate, almost half the activity remained in the glutamate after 4 min. A similar proportion of the total counts remained at or close to the origin and can be ascribed mainly to serine, alanine and glutamine [29]. The pretreatment with MP caused an almost 50 per cent fall in the relative incorporation into GABA. Following the injection of [U-^{14}C]pyruvate, a greater proportion of the total supernatant counts appeared in GABA (3.02 ± 0.43 per cent) and this was significantly reduced ($P < 0.01$) by pretreatment with MP to 0.79 ± 0.07 per cent. Although a greater proportion of the activity remained unaccounted for (unchanged pyruvate was not measured), this choice of precursor appears to provide a more sensitive test for the inhibition of GABA synthesis than does the use of [U-^{14}C]glutamate.

The results obtained here, which indicate a very rapid onset of the biochemical effects of MP following its i.p. injection are in agreement with those obtained earlier in which GAD activity alone was measured [19] and also with the observations of other workers using alternative species [30]. The latency of onset of seizures following i.p. administered MP is about 3 to 5 min [19, 30] and it would therefore appear that if MP is acting by interfering with the action of inhibitory GABA neurones, then newly synthesized GABA must be essential for maintaining an adequete supply of releasable GABA in the nerve terminal. The present methods therefore provide evidence of metabolic changes in the short term which are not readily detectable by other means.

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