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Valproate induced changes in GABA metabolism at the subcellular level

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The antiepileptic drug sodium valproate (VPA) is used primarily in the treatment of absence seizures but is also effective in the therapy of other forms of epilepsy [1]. Although the mechanism of action of VPA is unknown, it has been repeatedly demonstrated that VPA causes moderate elevations in brain GABA concentrations in rodents which correlate temporally with the onset and duration of anticonvulsant activity [2–8]. The GABA increase induced by VPA has been attributed to an inhibition of GABA-α-oxoglutarate aminotransferase (GABA-T; EC 2.6.1.19), the major GABA degradative enzyme [7] and, more recently, to an inhibition of succinic semialdehyde dehydrogenase (SSA-DH), the enzyme responsible for the subsequent stage in the GABA-shunt pathway [9–11]. Furthermore, VPA proved capable of activating glutamic acid decarboxylase *in vivo* (GAD; EC 4.1.1.15) [4, 5]. Thus, it was suggested that augmented CNS GABA would increase GABAergic inhibition and that this increase would explain the anticonvulsant action of VPA. However, since GABA concentrations and enzyme activities were determined in homogenates of whole brain or brain regions, it is not certain what portion of the increase in GABA produced by VPA is associated with nerve endings concerned with neurotransmission. GABA and its degradative enzymes are present in many cells including glial cells and neurons which do not use this amino acid as a neurotransmitter [12]. Thus, the cellular compartmentation of GABA makes it difficult to correlate elevation of whole brain GABA concentrations and neuropharmacological effects such as anticonvulsant action. More recently, Sarhan and Seiler [13], who examined GABA levels in subcellular fractions of brain tissue obtained from mice treated with VPA, found that VPA selectively increased GABA in a crude fraction of synaptosomes, which had been derived from nerve endings, and had little effect on GABA in non-synaptosomal elements. These experiments were confirmed indirectly by Iadarola and Gale [14], who demonstrated in rats in which the GABAergic afferent projection to the substantia nigra had been unilaterally destroyed that the GABA increase produced by VPA in this brain region is dependent upon the presence of nerve terminals. However, as in most other studies cited VPA was administered in high doses (300–400 mg/kg) whereas the effect of lower anticonvulsantly active doses of VPA on brain GABA concentrations is uncertain [10]. Furthermore, the mechanism by which VPA elevated GABA in nerve terminals remained unclear. The present report shows that VPA elevates GABA concentration in synaptosomes from mouse brain also at relatively low dose levels and that this effect can be attributed to an activation of GABA synthesis rather than to an inhibition of the degradation of GABA.

VPA (125, 200 and 290 mg/kg, respectively) was injected intraperitoneally to groups of 6 to 12 male NMRI-mice, weighing 25 to 30 g. Twelve untreated controls were used for each experiment. The respective doses were chosen on the basis of previous studies on the anticonvulsant potency of VPA [15] in which 30 min after administration the lowest dose (125 mg/kg) raised the electroconvulsant threshold in mice by 30 V (threshold in controls was 135 V), 200 mg/kg VPA raised the threshold by 50 V and 290 mg/kg was the ED_{50} as determined in the maximal electroshock seizure test [16]. In the present study, 30 min after administration of these VPA doses mice were decapitated and their brains either homogenized immediately in 4 ml ice-cold distilled water for the direct measurement of GABA, GAD and GABA-T or in 5 ml ice-cold 0.32 M sucrose, pH 7.0, for the further subcellular fractionation. The time from decapitation to homogenization never exceeded 30 sec. Under these conditions GABA levels were identical to those obtained after freezing the brains in liquid nitrogen [5]. Furthermore, the GABA content of synaptosomes is not subjected to change during the homogenization and fractionation procedures when the homogenization and all subsequent steps are carried out at 0–4° [17, 18]. Synaptosomal-enriched fractions were obtained by differential and Ficoll–sucrose density gradient centrifugation as described by Cotman [19] with some modifications. Wood and associates [17, 18] have shown that synaptosomes obtained by this procedure can be used as a model to evaluate drug-induced changes in GABA levels in nerve endings. In short, the homogenate was centrifuged at 1000 *g* for 5 min and the supernatant again centrifuged at 15,000 *g* for 12 min. The resultant pellet was resuspended in 5 ml 0.32 M sucrose and applied to a gradient consisting of 5 ml each of 4, 6, and 13% Ficoll in 0.32 M sucrose. After centrifugation at 63,580 *g* for 45 min the synaptosomal fraction was collected from the 6–13% interface. The fraction thus obtained was diluted with 4 volumes of 0.32 sucrose and centrifuged at 50,000 *g* for 20 min. The pellet was then resuspended in 2 ml redistilled water for the biochemical determinations. Examination of the pellet by electron microscopy, which was kindly performed by Prof. G. Böhme (Department of Histology at our School) revealed that more than 60 per cent of this fraction were synaptosomes, about 2 per cent were free mitochondria and about 35 per cent were not unequivocally assignable to either of these classes. GABA was determined in brain and synaptosomes by the radioreceptor-assay of Enna and Snyder [20] as adapted in our laboratory [21]. GAD activity was monitored by the method of Lowe *et al.* [22] in which the fluorescence of GABA formed during enzymatic incubation is measured. In a final volume of 1.0 ml, the incubation medium contained 5 mM L-glutamic acid, 0.24 mM pyridoxal phosphate, 100 mM phosphate buffer (pH 6.4) and 0.3 ml of the brain homogenate or 0.5 ml of the synaptosomal fraction, respectively. The activity of GABA-T was determined by measuring the rate of succinic semialdehyde formation by the method of Salvador and Albers [23]. In a final volume of 1.0 ml the incubation medium contained 10 mM of α-ketoglutaric acid, 50 mM of GABA and 0.2 ml of brain homogenate or the synaptosomal fraction, respectively. Details of both enzyme assays have been described in a previous paper [24]. Protein was measured by the procedure of Lowry *et al.* [25] as modified by Markwell *et al.* [26].

The results of the present experiments are shown in Fig. 1. In all doses administered, VPA produced significant increases in both brain and synaptosomal GABA of about 20 to 30 per cent compared to controls. This effect seemed to be independent of the dose administered. With respect

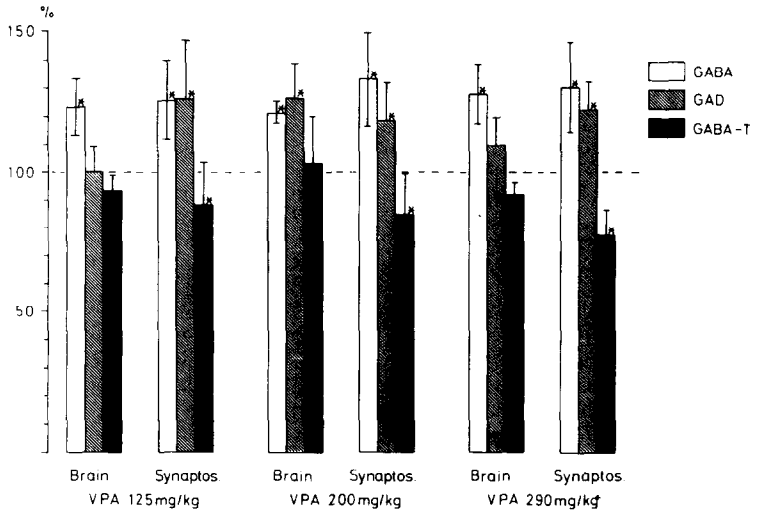

Fig. 1. Effect of sodium valproate (VPA) on the concentration of GABA and the activities of glutamate decarboxylase (GAD), and GABA-aminotransferase (GABA-T) in whole mouse brain and in synaptosomal fractions prepared from mouse brain 30 min after administration of VPA. Values represent the means of 6 to 12 mice ± S.D. in per cent of controls. Values significantly different ($P < 0.05$) from controls of the same day are marked by asterisks. Control GABA concentrations and enzyme activities (means ± S.D. of 30 determinations) were 2.06 ± 0.42 μmoles/g (GABA), 21.1 ± 2.0 μmoles/hr/g (GAD) and 43.4 ± 2.7 μmoles/hr/g brain (GABA-T) in whole brain and 8.9 ± 1.9 nmoles/mg protein (GABA), 400 ± 56 nmoles/hr/mg protein (GAD) and 1065 ± 138 nmoles/hr/mg protein (GABA-T) in the synaptosomal enriched fraction, respectively. Protein content of the latter fraction was 5.2 ± 0.52 mg/g brain.

to GAD and GABA-T activities, differences were observed between whole brain and synaptosomes. In whole brain, 125 and 290 mg/kg VPA had no significant effect on GAD, whereas 200 mg/kg activated the enzyme by 27 per cent. GABA-T was not affected by any of the three VPA doses studied. This is in agreement with previous studies [4] in which whole brain GAD was only activated by non-toxic doses of VPA which had no effect on GABA-T. In the synaptosomal fraction, the GAD activity was elevated by all doses of VPA by about 20 to 26 per cent and GABA-T was slightly but significantly inhibited. Whereas the activation of GAD seemed to be maximal with the lowest dose of VPA used, GABA-T was inhibited in a dose-dependent manner. The present results confirm that VPA increases GABA in nerve terminals, i.e. the pool of GABA which can be mobilized for synaptic transmission. Since decreases in GABA concentrations in nerve terminals of only 28 per cent have been demonstrated to impair the functioning of the inhibitory transmitter to such an extent that seizures occur [18], the modest potency of VPA to increase synaptosomal GABA is sufficient to predict anticonvulsant activity. The elevation of synaptosomal GABA by VPA could be attributed to a concomitant increase in the activity of GAD, which is known to be preferentially localized in the nerve endings [12], and is believed to be the rate limiting enzyme that normally determines the steady-state levels of GABA in brain [27]. Inhibition of GABA-T in synaptosomes by VPA was only moderate if one takes into consideration that *in vivo* there is a spare capacity of this enzyme of approximately 50 per cent [28]. The inhibition of GABA-T observed cannot be the result of a direct effect of VPA on this enzyme. The previous claim that VPA is a competitive inhibitor of GABA-T with a K_i of 1.4 mM [7] is probably erroneous and arose because the assay system employed a linked reaction including SSA-DH [10]. More recent experiments had shown that VPA is a poor inhibitor of GABA-T with K_i values ranging from 23 mM [11] to 87 mM [24]. Such concentrations were most certainly not achieved in mouse brain after administration of VPA in the doses as used in this study. However, VPA is a far more potent inhibitor of SSA-DH with a K_i of 0.5 mM for rat brain [11] and a value below 1 mM for mouse brain [10]. Inhibition of this enzyme will result in accumulation of SSA which had been shown to exert a strong inhibitory effect on the GABA-T forward reaction [11]. Thus, in doses as used in this study, VPA could act indirectly as an inhibitor of GABA-T. With respect to synaptosomal GAD, our results suggest that this enzyme can only be activated to a certain degree and this degree was reached by the lowest VPA dose used. Higher doses caused no further activation of GAD and thereby no further increase in GABA concentrations though the anticonvulsant effect increased. With respect to this lack of correlation between the increase in synaptosomal GABA and anticonvulsant activity it has to be considered whether the anticonvulsant effect of VPA may depend not only on its ability to elevate GABA in nerve terminals but also on the regional location of the nerve terminals in which the GABA increase takes place. It has been previously reported that different brain areas show different degrees of change in GABA after VPA [2, 8]. This suggests a dose dependency in the VPA induced elevation of synaptosomal GABA in certain discrete brain regions which may have been overlooked when determining GABA and related enzymes in synaptosomes from whole brain.

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Cytochrome P-450-dependent metabolism of 1,1,2,2-tetrachloroethane to dichloroacetic acid *in vitro*

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During the past decade, it has become increasingly evident that many of the adverse effects of halogenated hydrocarbons, which include liver and kidney damage [1] and cancer [2], can be attributed to reactive intermediates of the parent compound formed during its metabolism by the cytochrome P-450-dependent monooxygenase system [3]. In recent years, considerable interest has been devoted to elucidating the mechanisms by which chlorinated ethylenes such as vinyl chloride and trichloroethylene are activated to toxic intermediates [4]. The chlorinated ethanes, which are less widely used [1, 5] in part due to higher acute toxicity, have received less attention.

1,1,2,2-Tetrachloroethane (tetrachloroethane) has been shown recently to be a liver carcinogen in mice, whereas the bioassay provided no convincing evidence for carcinogenic potential in rats [6]. Tetrachloroethane is hepatotoxic in several species including man [5], and it is genetically active in three different test systems including the Ames test [7, 8]. The species difference in carcinogenic potential and the lack of direct cytotoxicity of tetrachloroethane toward Ehrlich–Landschütz diploid ascites tumor cells [9] are consistent with a requirement for metabolic activation of the compound. Tetrachloroethane metabolism has been demonstrated *in vitro* by monitoring dechlorination [10] and substrate disappearance [11], but in neither study were any metabolites identified. At present, little information is available on the pathways by which toxic intermediates might be formed.

The major urinary metabolites found upon *in vivo* administration of tetrachloroethane to mice are dichloroacetic acid (DCA), trichloroethanol, and trichloroacetic acid, the latter two apparently formed via oxidation of trichloroethylene, a decomposition product of tetrachloroethane in aqueous media. DCA was postulated to form by oxidation of dichloroacetaldehyde [12]. However, in light of recent results obtained with chloroform [13], dichloromethane [14], and chloramphenicol [15], an equally attractive pathway for the formation of DCA is hydrolysis of dichloroacetyl chloride; this could be formed by a cytochrome P-450-dependent hydroxylation of tetrachloroethane to give $CHCl_2C(OH)Cl_2$, followed by spontaneous dehydrochlorination to give the acyl chloride, $CHCl_2COCl$.

Tetrachloroethane has been shown to give a type I difference spectrum with liver microsomes from phenobarbital-treated rats [16], and phenobarbital is known to stimulate the metabolism of a number of related compounds [13–15]. Therefore, we have examined the metabolism of tetrachloroethane by intact liver microsomes and a reconstituted monooxygenase system from phenobarbital-treated rats.