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GABA DEHYDROGENASE ACTIVITY IN RAT BRAIN

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Abstract—The crude mitochondrial fraction of rat brain contains an active dehydrogenase involved in the direct oxidation of γ-aminobutyric acid. INT (*p*-iodonitrotetrazolium violet) can serve as an efficient acceptor of electrons in this dehydrogenase reaction. During this oxidation of GABA, ammonia is not produced. *In vitro* the dehydrogenase activity is inhibited by certain MAO inhibitors. The effects of various inhibitors of GABA-T and GAD were also investigated. The dehydrogenase activity was found to be susceptible to various anti-convulsants and inhibitors of electron transport. The co-factors which may be involved in the transfer of electrons during GABA oxidation in the presence of INT are also discussed.

The metabolic degradation of GABA (γ-aminobutyric acid), an inhibitory neurotransmitter supposed to be involved in Huntington's disease, tardive dyskynesia, Parkinsonism and epileptic seizures [1–6], is believed to occur primarily through the GABA-shunt pathway [7–10], although evidence for its direct oxidation has also been reported [11–16]. However, Bacila *et al.* [17] failed to confirm the observations of Sacktor *et al.* [15]. Much attention has been paid to the role of GABA-T (EC 2.6.1.19), succinic semialdehyde dehydrogenase (SSADH; EC 1.2.1.24) and NADPH-dependent aldehyde reductase (EC 1.1.1.2) to explain the mode of action of anti-convulsants [9, 18–20]. The present work demonstrates the occurrence of a very active GABA dehydrogenase system in the crude mitochondrial fraction of rat brain which appears to be responsible for the direct oxidation of GABA in brain. Employing a tetrazolium salt as the electron acceptor, an assay system for GABA dehydrogenase has been developed and some of the properties of the GABA–tetrazolium reductase system and the possible co-factors involved in the transfer of electrons during GABA oxidation are presented in this communication.

MATERIALS AND METHODS

The crude mitochondrial fraction of whole brain of male albino rats (175 g) was prepared according to the method of Sacktor *et al.* [15] and the respiratory activity of such preparations in the presence of GABA was also determined in a medium described by Sacktor *et al.* [15]. In order to determine whether GABA oxidation is mediated by a dehydrogenase system, preliminary experiments indicated that INT [2(*p*-iodophenyl)-3-*p*-nitrophenyl-5-phenyltetrazolium chloride; *p*-iodonitrotetrazolium violet] can serve as an efficient electron acceptor. The reaction mixture for the dehydrogenase activity contained 0.02 M Tris–HCl buffer, pH 8.5, 0.02 M GABA, 500 μg INT and an aliquot of mitochondrial suspension equivalent to 3–5 mg of protein in a final volume of 2 ml. The reaction mixture containing the enzyme with or without potential inhibitors was first preincubated for 5 min at 37° followed by the addition of GABA and INT. The reaction was stopped after 5 min by the addition of 1 ml of 10% trichloroacetic acid and the formazan was extracted with ethyl acetate and measured at 520 nm [21]. Preliminary experiments indicated that the pH of the assay medium and concentrations of GABA and INT used were optimal for the enzyme activity. The activity at pH 7.0 and 7.5 was 25 and 33% respectively of the activity at pH 8.5. The enzyme activity was also measured under conditions where the rate of reaction is linear both as a function of time of incubation and enzyme concentration. Protein was determined according to the method of Lowry *et al.* [22]. All values are corrected for appropriate controls.

GABA, INT, INT-formazan, rotenone, iproniazid, tranylcypromine, diphenylhydantoin, antimycin A, malonate, oxaloacetate, amino-oxyacetate, ADP, AMP, pyridoxal phosphate, Tris-buffer, NAD, NADP, acetazolamide, amytal, thenoyltrifluoroacetone and chlorpromazine were obtained from Sigma Chemical Co. (St. Louis, MO). Clorgyline and deprenyl were gifts from May & Baker Ltd (Dagenham, U.K.) and Professor J. Knoll (Semmelweiss University, Budapest), respectively. Pargyline and catron were obtained from Abbott Laboratories (North Chicago, IL) and Lakeside Laboratories (Cincinatti, OH), respectively. Sodium valproate and Lilly 51641 were gift samples from Reckitt & Colman (Hull, U.K.) and Lilly Research Laboratories, Eli Lilly and Co. (Indianapolis, IN), respectively. *N*-Ethylmaleimide and sodium azide were obtained from Pierce Chemical Co. (Rockford, IL). Isoniazid was obtained from Fluka AG (Bern, Switzerland). Phenobarbitone and diazepam were gift samples from Central Drug Laboratories (Calcutta, India). All other reagents were of analytical grade wherever possible.

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RESULTS AND DISCUSSION

The results presented in Table 1 show that certain MAO inhibitors like iproniazid, tranylcypromine, clorgyline and deprenyl inhibited GABA-INT reductase activity at high concentrations (0.1–0.01 mM) whereas catron, pargyline and Lilly 51641 were without any effect. In contrast, Baxter and Roberts [23] found that iproniazid (1 mM) had no effect on GABA-T, although certain hydrazine MAO inhibitors have been reported to inhibit GABA-T [24]. It is, however, not clear how these MAO inhibitors produce inhibition of GABA oxidation. It was further observed that the mitochondrial preparation in the presence of GABA and INT or air as the electron acceptor failed to show any production of ammonia. The microdiffusion technique of Conway and Byrne [25], the direct estimation of ammonia by the method of Bradford *et al.* [26] or the use of an ammonia electrode all failed to reveal the formation of any extra ammonia during GABA dehydrogenase reaction. Chromatographic analysis revealed that the product of GABA oxidation is γ-amino-β-hydroxybutyrate. γ-Aminocrotonic acid may, however, be an unstable intermediate in this process [15].

The dehydrogenase showed optimal activity at pH 8.5 and is not very stable. Deep-frozen preparations (−20°) lost most of their activity within 24 hr which could not be restored upon addition of phenazine methosulphate, NAD or pyridoxal phosphate but NADP could restore some of the INT-reductase activity. Hence the experimental results presented here were performed with freshly prepared crude mitochondrial preparations, unless stated otherwise. The dehydrogenase activity of freshly prepared crude mitochondria was found to be stimulated by NADP. The dehydrogenase activity was highly stimulated in the presence of ADP and AMP (1 and 0.5 mm) but pyridoxal phosphate had no effect. Heat-treated mitochondrial preparations, however, failed to reduce INT in the presence of GABA. Employing the manometric technique, when a crude mitochondrial fraction was allowed to respire in the presence of GABA, the oxygen uptake data confirmed the observations of Sacktor *et al.* [15], so far as the effects of ADP, malonate, amytal and antimycin A are concerned. It was also observed that amino-oxyacetate (0.1 mM) caused 25% inhibition of oxygen uptake of such preparations in the presence of GABA whereas at 0.01 mM concentration the inhibition was only 12%. At such concentrations of amino-oxyacetate, it is known that GABA-T is almost completely inhibited [23].

The effects on GABA-INT reductase of certain inhibitors, especially of GABA-T and GAD (EC 4.1.1.15), are shown in Table 2. The inhibitory effects of some of these inhibitors on GABA-T and GAD are explained on the basis of their interaction with pyridoxal phosphate, a co-factor required by these enzymes or on the basis of their resembling GABA in chain length and charge separation [20, 23, 27]. However, the lack of specificity of these inhibitors makes interpretation of results difficult. Both malonate and oxaloacetate inhibited GABA-INT reductase, the former being found to have no effect on GABA-T [23]. It is known that malonate and oxaloacetate are competitive inhibitors of succinic dehydrogenase [28, 29] and it is possible that these compounds react with GABA dehydrogenase directly.

All the anti-convulsants tested were found to inhibit GABA dehydrogenase (Table 3). Baxter and Roberts [23] reported that acetazolamide and diphenylhydantoin do not affect GABA-T. However, recent reports indicate that various branched-chain fatty acids including sodium val-

Table 1. Effects of MAO inhibitors on GABA-INT reductase activity

Inhibitors	Final concentration (mM)	μmoles INT formazan formed/mg of protein/hr (±S.D.)	Per cent inhibition
None	—	117 ± 9.6 (10)	—
Iproniazid	0.1	84 ± 6.4 (6)	28
	0.01	99 ± 8.2 (6)	15
Tranylcypromine	0.1	89 ± 6.2 (6)	24
	0.01	90 ± 5.8 (4)	23
Clorgyline	0.1	84 ± 5.2 (6)	28
	0.01	98 ± 6.4 (4)	16
Deprenyl	0.1	60 ± 5.2 (6)	49
	0.01	80 ± 4.6 (4)	32
Catron	0.1	117 ± 8.2 (4)	0
	0.01	116 ± 6.8 (4)	0.9
Pargyline	0.1	117 ± 7.5 (4)	0
	0.01	117 ± 7.2 (4)	0
Lilly 51641	0.1	116 ± 7.4 (4)	0.9
	0.01	117 ± 6.4 (4)	0

The incubation mixture contained 0.02 M Tris–HCl buffer, pH 8.5, 0.02 M GABA, 500 μg INT and an aliquot of mitochondrial suspension equivalent to 3–5 mg of protein in a final volume of 2 ml. The inhibitors were first preincubated with the enzyme for 5 min at 37° followed by addition of GABA and INT. The reaction was stopped after 5 min and the formazan was extracted with ethyl acetate and measured at 520 nm [21]. Protein was determined according to the method of Lowry *et al.* [22]. Figures in parentheses denote the number of experiments. All values are corrected for appropriate blanks.

Table 2. Effects of various compounds on GABA-INT reductase activity

Addition	Final concentration (mM)	μmoles INT formazan formed/mg of protein/hr (±S.D.)	Per cent inhibition
None	—	117 ± 9.6 (10)	—
Amino-oxyacetate	0.01	25 ± 2.5 (6)	79
	0.001	89 ± 5.4 (6)	24
Malonate	1.0	28 ± 2.8 (6)	76
	0.1	57 ± 3.5 (6)	52
Oxaloacetate	1.0	69 ± 4.5 (6)	41
	0.1	89 ± 5.2 (4)	24
Thiosemicarbazide	1.0	0 (4)	100
	0.1	55 ± 3.4 (4)	53
Semicarbazide	1.0	68 ± 3.6 (5)	42
	0.1	92 ± 5.2 (5)	21
Isoniazid	1.0	21 ± 1.2 (6)	82
	0.1	75 ± 4.6 (4)	36
N-Ethylmaleimide	1.0	4 ± 1.8 (6)	97
	0.1	16 ± 2.1 (4)	86

Experimental details are the same as for Table 1. Figures in parentheses denote the number of experiments.

proate inhibit GABA-T, GAD, SSADH and aldehyde reductase [30–37]. Although it is too early to speculate on any correlation between inhibition of GABA oxidation and the anti-convulsant action of these drugs, these results open up a new direction for investigating the mode of action of these drugs in relation to their anti-convulsant action. On the other hand, a possibility exists that oxidation of GABA may have some relation with seizure activity.

The results presented in Table 4 indicate that GABA-INT reductase is highly susceptible to inhibition by thenoyltrifluoroacetone, and the inhibition produced by amytal and rotenone coupled with the stimulatory effect produced by NADP indicates the possible involvement of an alternative pathway of electron transfer involving reduced pyridine nucleotide dehydrogenase system. It is, however, difficult to say at this stage whether the oxidation of NADPH involves NADPH oxidase or a transhydrogenase system coupled with NADH oxidase. There are at least two sites for INT to accept electrons [38], one of which is antimycin A sensitive while the other site is at the level of cyt *c* which is chlorpromazine-sensitive [38]. The effects of cyanide, azide and anaerobiosis are quantitatively similar and suggest that these inhibitors also act at the level of cyt a/a_3 [38, 39] and these results indicate that this may also be another site for INT to accept electrons. Since anaerobiosis produces about 50% inhibition of INT reduction, it appears that the presence of oxygen is necessary for optimal activity of the dehydrogenase so far as tetrazolium reduction is concerned.

GABA dehydrogenase appears to be a respiratory enzyme [15, 16] whose activity depends upon the structural integrity of the mitochondria, since deep-frozen preparations lose most of their activity within 24 hr. The effects of semicarbazide, iproniazid, anti-convulsants, azide, cyanide, oxaloacetate, malonate and anaerobiosis coupled with the observation that the activity is stimulated by NADP, ADP

Table 3. Effects of some anti-convulsants on GABA-INT reductase activity

Anti-convulsants	Final concentration (mM)	μmoles INT formazan formed/mg of protein/hr (±S.D.)	Per cent inhibition
None	—	117 ± 9.6 (10)	—
Phenobarbitone	5.0	63 ± 4.2 (6)	46
	1.0	78 ± 4.8 (6)	33
	0.1	88 ± 4.6 (6)	25
Diphenylhydantoin	1.0	33 ± 2.5 (6)	72
	0.1	71 ± 4.6 (6)	41
Diazepam	2.0	33 ± 3.2 (6)	72
	1.0	65 ± 4.4 (6)	44
Valproate	1.0	61 ± 3.5 (6)	48
	0.1	71 ± 4.0 (6)	41
Acetazolamide	2.0	62 ± 4.0 (6)	47
	1.0	76 ± 4.2 (6)	35
	0.1	84 ± 5.4 (6)	28

Experimental details are the same as for Table 1. Figures in parentheses denote the number of determinations.

Table 4. Effects of inhibitors of electron transport on GABA-INT reductase activity

Inhibitor	Final concentration (mM)	μmoles INT formazan formed/mg of protein/hr (±S.D.)	Per cent inhibition
None	—	117 ± 9.6 (10)	—
Thenoyltrifluoroacetone	1.0	14 ± 1.6 (6)	88
	0.1	18 ± 2.1 (6)	85
	0.01	28 ± 2.5 (6)	76
Amytal	0.5	0.8 ± 0.22 (6)	99
	0.1	70 ± 3.6 (6)	40
Rotenone	1.0	0 (4)	100
	0.1	26 ± 3.5 (6)	78
	0.01	65 ± 4.2 (6)	44
Antimycin A (μg)	5.0	65 ± 5.2 (6)	44
	10.0	39 ± 3.1 (6)	67
	15.0	22 ± 4.2 (6)	81
	25.0	5 ± 1.2 (6)	96
Chlorpromazine	1.0	1.8 ± 0.8 (6)	98
	0.1	94 ± 7.2 (6)	20
Hydroxylamine	0.1	0 (4)	100
	0.01	12 ± 1.5 (6)	90
Cyanide	1.0	53 ± 4.6 (6)	55
	0.1	86 ± 5.2 (6)	26
Azide	1.0	58 ± 4.2 (6)	50
	0.1	79 ± 6.2 (4)	32
Anaerobiosis	—	57 ± 8.7 (6)	51

Experimental details are the same as for Table 1. Anaerobiosis was effected by evacuating the system for 10 min at 4° using the Thunberg tubes. Figures in parentheses represent the number of experiments.

and AMP but not by pyridoxal phosphate clearly distinguishes it from the enzymes of the GABA-shunt pathway. Moreover, it may be presumed that washed mitochondria contain little, if any, 2-oxoglutarate for GABA-T activity, since the K_m value for 2-oxoglutarate appears to be 4×10^{-3} M [23]. On the other hand, the endogenous concentration of succinic semialdehyde in the brain is also very low [19]. Hence it is most unlikely that washed mitochondria can cause any tetrazolium reduction by SSADH under the present experimental conditions. Work is in progress to determine the exact site from which the electrons are removed from GABA as well as the exact localisation of the enzyme, although Sacktor *et al.* [15] reported that the first product of oxidation of GABA is γ-aminocrotonic acid.

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