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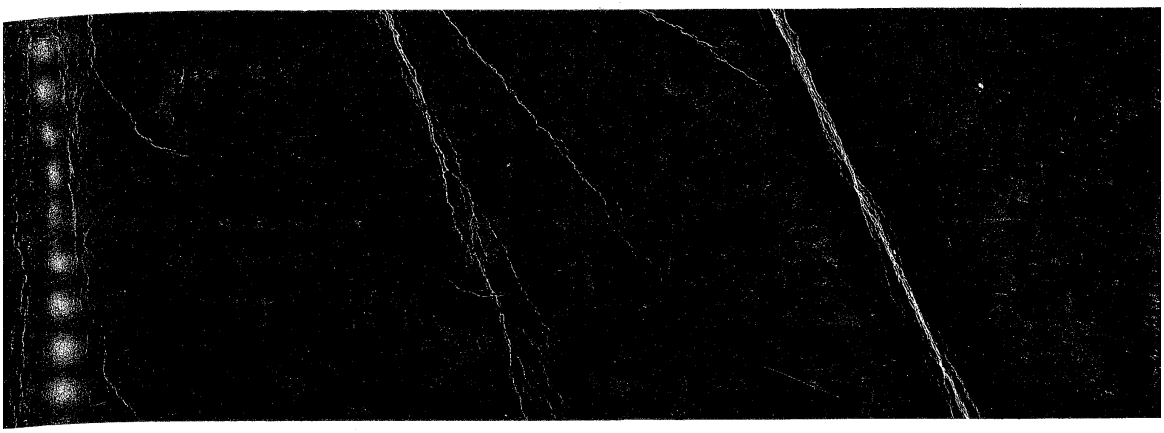

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DI-n-PROPYLACETATE AND GABA DEGRADATION. PREFERENTIAL INHIBITION OF SUCCINIC SEMIALDEHYDE DEHYDROGENASE AND INDIRECT INHIBITION OF GABA-TRANSAMINASE

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Abstract—The kinetic constants for 4-aminobutyrate: 2-oxoglutarate aminotransferase (GABA-transaminase) and succinate-semialdehyde: NAD^+ oxidoreductase (SSA-DH) have been determined using rat brain homogenate.

The Michaelis constants for GABA-T at saturated substrate concentrations were calculated to be K_{GABA} = 1.5 mM, $K_{2\text{-}OG}$ = 0.25 mM, $K_{Gl.U}$ = 620 μM, and K_{SSA} = 87 μM. The V_{max} for the reaction using GABA and 2-oxoglutarate (2-OG) as substrates (forward reaction) was found to be 35.2 μmol/g/h, whereas for the reaction using glutamate (Glu) and succinate-semialdehyde (SSA) a value of 63.0 μmol/g/h was found.

The kinetics of GABA-T have been shown to be consistent with a Ping Pong Bi Bi mechanism. Substrate inhibition of the forward reaction, through formation of a dead-end complex, was found to occur with 2-OG (K_i = 3.3 mM), whereas GABA was found to be a product inhibitor of the reverse reaction (K_i = 0.6 mM). Using the appropriate Haldane relationship, a K_{eq} of 0.04 for GABA-T was found, indicating that the reaction was strongly biased towards GABA.

For SSA-DH, the K_m of SSA was determined (9.1 μM) and the V_{max} was 27.5 μmol/g/h. The effect of di-n-propylacetate (DPA) on both GABA-T and SSA-DH was measured. DPA inhibited SSA-DH competitively with respect to SSA, giving a K_i of 0.5 mM. GABA-T was only slightly inhibited. The K_i of DPA for the forward reaction was 23.2 mM with respect to GABA, which was 40–50 times higher than that for SSA-DH. For the reverse reaction the K_i of DPA was found to be nearly the same (15.2 mM with respect to Glu and 22.9 mM with respect to SSA). These results suggest that GABA accumulation in the brain, after administration of DPA *in vivo*, is caused by SSA-DH inhibition. Two mechanisms are indicated by the data. (1) The higher level of SSA, which results from inhibition of SSA-DH, initiates the reverse reaction of GABA-T, thus increasing the level of GABA via conversion of SSA. (2) The degradation of GABA is inhibited by SSA, since SSA has a strong inhibitory effect on the forward reaction, as calculated from the present data.

IN MAMMALIAN brain gamma-aminobutyric acid (GABA) is thought to be an inhibitory neurotransmitter (KRNJEVIĆ, 1970). A decrease in the concentration of GABA has been implicated in the onset of convulsions (TOWER, 1976). Accordingly, it has been reported by many authors that several anti-convulsant drugs increase GABA levels in the brain through inhibition of its degradation (See TAPIA, 1975).

Elevation of GABA levels has been extensively studied by administration of drugs which affect the activity of 4-aminobutyrate: 2-oxoglutarate aminotransferase (EC 2.6.1.19; GABA-T). Such drugs include aminooxyacetic acid (AOAA; BAXTER & ROBERTS, 1961), ethanolamine-*O*-sulphate (EOS; FOWLER & JOHN, 1972), γ-acetylene-GABA and γ-vinyl-GABA (SCHECHTER *et al.*, 1977; JUNG *et al.*, 1977). Similarly, di-n-propylacetate (Depakine®, Valproate, DPA) also increases the concentration of GABA in the brain (ANLEZARK *et al.*, 1976; GODIN *et al.*, 1969; SIMLER *et al.*, 1973) and its action has been ascribed to an inhibition of GABA-T (GODIN *et al.*, 1969; SIMLER *et al.*, 1973; FOWLER *et al.*, 1975). However, some authors reported a preferential action of DPA on SSA-DH (HARVEY *et al.*, 1975; ANLEZARK *et al.*, 1976). Two specific reactions are involved in

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Abbreviations used: GABA-T, 4-aminobutyrate: 2-oxoglutarate aminotransferase (EC 2.6.1.19); SSA-DH, succinate-semialdehyde: $NAD(P)^+$ oxidoreductase (EC 1.2.1.16); Glu, glutamate; SSA, succinate-semialdehyde; 2-OG, 2-oxoglutarate; SA, succinate; DPA, di-n-propylacetate; K, Michaelis constant under saturated conditions; K_m apparent Michealis constant under non-saturated conditions; V, maximum velocity under saturated conditions; V_{max}, apparent maximum velocity at a fixed non-saturating concentration of a single substrate.

the degradation of GABA; firstly, the reversible conversion of GABA and 2-oxoglutarate (2-OG) into succinate-semialdehyde (SSA) and glutamate (Glu), respectively, which is catalysed by GABA-T, and secondly, the subsequent oxidation of SSA to form succinate by succinate-semialdehyde: NAD^+ oxidoreductase (EC 1.2.1.16; SSA-DH) using NAD as a cofactor, which reaction is almost irreversible. Since the equilibrium of the GABA-T catalysed reaction is biased towards GABA (DUFFY *et al.*, 1972), inhibition of SSA-DH may also result in elevation of the GABA concentration. However, this possibility has so far been neglected, because SSA-DH was considered to be very efficient in preventing accumulation of SSA. Recent studies have suggested that the activity of SSA-DH can be affected by mono- and bivalent cations like sodium, potassium and calcium (DE BOER & BRUINVELS, 1977*b*). Therefore, it is possible that SSA-DH may play a regulating role in GABA degradation *in vivo* and that inhibition of this enzyme might increase GABA concentrations through activating the reverse reaction of GABA-T.

The aim of the present paper has, therefore, been to study this regulatory role of SSA-DH in relation to the action of DPA. The affinity of SSA for both GABA-T and SSA-DH and its alteration by DPA have been determined as well as the effect of DPA on the conversion of GABA into SSA.

MATERIALS AND METHODS

Materials. GABA was obtained from Calbiochem, Los Angeles, CA. Triton X-100, 2-oxoglutaric acid, di-sodium succinate and 2-mercaptoethanol were obtained from BDH Chemicals Ltd. Pyridoxamine-di-HCl and SSA were purchased from Sigma Chemical Company. NAD and NADH were obtained from Boehringer-Mannheim. [U^{14}C]GABA (specific radioactivity 4.6 mCi/mmol) was obtained from the Radiochemical Centre, Amersham, U.K. All other chemicals were purchased from Merck, Darmstadt. DPA was a gift of Labaz B.V., Maassluis, The Netherlands.

Tissue preparation. Randomly selected male Wistar rats (125–175 g) were killed by decapitation and their brains were rapidly removed and weighed. A 10% (w/v) brain homogenate was prepared in an ice-cold solution containing 0.32 M-sucrose and 4.5 mM-2-mercaptoethanol, using a Teflon–glass Potter–Elvehjem homogenizer. One volume of the homogenate was added to 3 vol of ice-cold Triton medium (0.67% w/v Triton X-100, 50 mM-Tris–HCl (pH 8.5) and 4.5 mM-2-mercaptoethanol) and kept on ice-water for 1 h before use.

GABA-T assay using GABA as substrate (forward reaction). For the performance of the assay of the GABA-T forward reaction a radiochemical method was used as previously described (DE BOER & BRUINVELS, 1977*a*) with the following modifications. NAD was omitted from the incubation mixture, unless otherwise specified, to exclude interference with SSA-DH, since DPA might affect this enzyme too. A control was carried throughout the whole procedure in the presence of NAD but without DPA. Tissue concentration was 4.44 mg/ml (wet wt.). After precincubation at 22°C for 30 min the reaction was started by the addition of homogenate and the mixture was incubated for a further 30 min at the same bath temperature. All experiments were carried out in duplicate.

GABA-T assay using SSA as substrate (reverse reaction). The reaction was carried out under similar conditions to the forward reaction. The incubation mixture contained 50 mM-Tris–HCl (pH 8.5), 20 mM-2-mercaptoethanol and 0.12 ml Triton-treated homogenate in a total volume of 0.615 ml. The final tissue concentration was 4.44 mg/ml. The reaction mixture was preincubated at 22°C for 1 h in the presence of glutamate (0.5–5.0 mM) and in the absence of SSA. The reaction was started by addition of 60 μl SSA solution, resulting in final concentrations of 50–600 μM-SSA. For the inhibition studies DPA was added simultaneously with SSA.

Since the sodium salt of DPA was used throughout this study, a solution in which DPA was replaced by sodium-isethionate, equimolar to the highest DPA concentration, was used as control.

After an incubation period of 15 or 20 min, the reaction was terminated by adding an equal volume of ice-cold 5% TCA solution. Subsequently, the tubes were placed in ice-water. After centrifugation (10 min × 1200 *g*) the concentration of 2-OG in 0.5 ml of the supernatant was determined in duplicate.

Determination of 2-oxoglutarate. The fluorometric determination of α-oxo acids as described by TAKEDA *et al.* (1976) was used. To neutralize 0.5 ml of the 2.5% TCA solution (sample solution), 0.2 ml of a solution containing 2 vol 1 M-Tris–HCl (pH 8.3) and 1 vol 1 M-NaOH was added. Thereafter, 5 ml of a solution containing 2% (v/v) pyridine in methanol, 100 mg/l pyridoxamine–di-HCl and 2.0 g/l zinc acetate was added. The reaction of pyridoxamine, 2-oxoglutarate and Zn(II) ion was found to yield a Zn(II) chelate of the aldimine (MATSUSHIMA & MARTELL, 1967). After incubation for 30 min at 37°C the reaction mixture was cooled in ice-water for 3–5 min. The fluorescence intensity was measured within 2 h at room temperature using a Baird-Atomic spectrophotofluorometer with excitation at 395 nm and emission at 475 nm. Standards and blanks were carried throughout the entire assay procedure. Blanks contained the complete incubation mixture, except SSA. The standards were prepared in the same way as blanks and 2-OG was added to final concentrations of 20–80 μM. The change in the glutamate concentration used in the GABA-T assay resulted in blanks which varied to a maximum of 20% and this variation was proportional to the glutamate concentration. Therefore, the assay was always performed with standards of all glutamate concentrations used. Incubation mixtures, to which the TCA solution was added immediately after starting the reaction by addition of SSA, were used as additional controls. When 2-OG was incubated under the conditions of the assay in the absence of SSA it was not significantly metabolized, as measured after 1 h (recovery 98.4 ± 1.3%; n = 13).

SSA-DH assay. Measurement of the SSA-DH activity was performed as described previously (DE BOER & BRUINVELS, 1977*a*), using a tissue concentration of 0.44 mg/ml (wet wt.).

Data analysis. The apparent kinetic parameters of the forward reaction of GABA-T were calculated from Lineweaver–Burk plots.

Since in both the assay of the reverse reaction of GABA-T and in the assay of SSA-DH more than 5% of the initial concentration of SSA was converted, the apparent kinetic constants were calculated using the integrated form of the Henri–Michaelis–Menten equation (SEGEL, 1975).

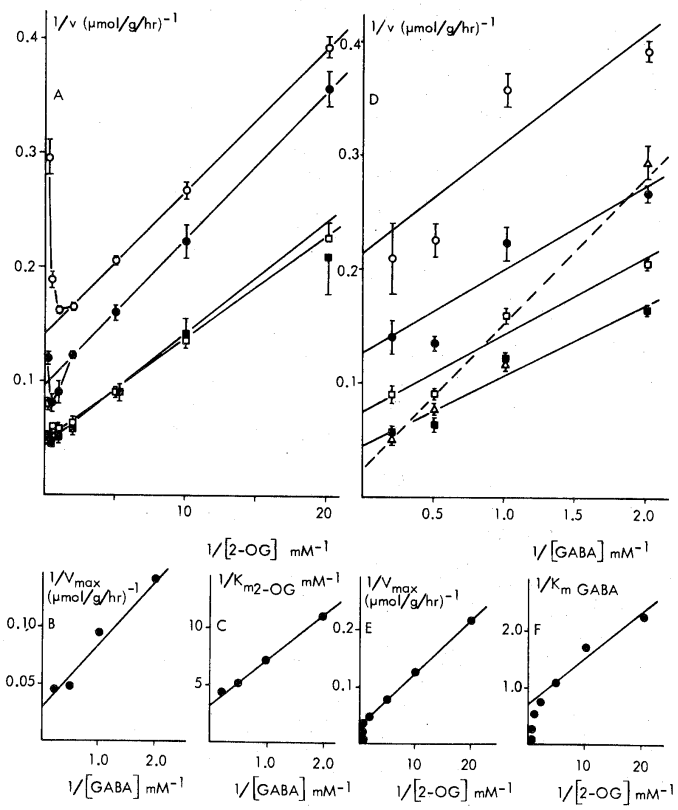

FIG. 1. The kinetic constants of the GABA-T forward reaction. Incubations were performed in 50 mM-Tris–HCl (pH 8.5)–20 mM-2-mercaptoethanol and in the presence of Triton X-100 at a temperature of 22°C. A and D are Lineweaver–Burk plots, of the mean of three experiments, whereas B, C, E and F are replots of the reciprocal apparent kinetic constants obtained from A and D to calculate the limiting Michaelis constants. The reciprocal fixed substrate concentration is given on the horizontal axis. The equations which were applied to calculate the constants are given in the legend to Table 1. The resulting values are summarized in Table 1. A. Reciprocal plot of the velocity vs the concentration of 2-OG with GABA as a fixed substrate. GABA concentrations: ○——○, 0.5 mM; ●——●, 1.0 mM; □——□, 2.0 mM; ■——■, 5.0 mM. B. Replot of reciprocal apparent maximum velocity vs the reciprocal GABA concentration. C. Replot of the reciprocal apparent K_m for 2-OG vs the reciprocal GABA concentration. D. Reciprocal plot of the velocity vs the concentration of GABA with 2-OG as fixed substrate. 2-OG concentrations: ○——○, 0.05 mM; ●——●, 0.1 mM; □——□, 0.2 mM; ■——■, 0.5 mM; △——△, 6.0 mM. The broken line for 6.0 mM-2-OG indicates that substrate inhibition occurred. For reasons of simplicity the curves obtained with 2-OG concentrations of 1.0 mM and 2.0 mM are not shown in the figure. E. Replot of the reciprocal apparent maximum velocity vs the reciprocal 2-OG concentration. F. Replot of the reciprocal apparent K_m for GABA vs the reciprocal 2-OG concentration.

To obtain limiting Michealis constants for the substrates of GABA-T from the apparent constants the equations for a Ping Pong Bi Bi mechanism (CLELAND, 1963) were applied, since it is generally accepted that transaminations occur via such a mechanism (MEISTER, 1955; SCOTT & JAKOBY, 1959; HENSON & CLELAND, 1964).

The type of inhibition by DPA of GABA-T was determined graphically. The K_i's were calculated from a replot of the ratio of the apparent K_m and V_{max} vs concentration of inhibitor as suggested by SEGEL (1975) for mixed-type inhibition.

All constants were calculated from the plots using the least squares method. Variance analysis for regression lines was used to test the significance of differences in K_m and V_{max}.

RESULTS

Kinetics of GABA-T

Reaction with GABA as substrate (forward reaction). The kinetic constants of the GABA-T forward reaction were determined using concentrations of 0.5–5.0

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