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CYTOSPECTROPHOTOMETRIC INVESTIGATION OF GABA-TRANSAMINASE IN THE RAT CEREBELLAR CORTEX

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A modification of a histochemical method for the detection of GABA-transaminase is suggested. Optimal concentrations of substrates and coenzymes were chosen on the basis of an investigation of the enzyme reaction in frozen sections through the rat cerebellar cortex by a quantitative microspectrophotometric method.

KEY WORDS: *GABA-transaminase, cytospectrophotometry; kinetics of histochemical reaction; cerebellar cortex.*

A number of investigations into the connection between GABA, the role of which in inhibition has been studied in detail [4, 10], and the regulation of behavior and the action of various psychotropic drugs have been published in recent years [3, 5, 14]. In 1965 Van Gelder suggested a histochemical method for the detection of GABA-transaminase (GABA-T; 4-aminobutyrate: 2-oxoglutarate aminotransferase, EC 2.6.1.19), the principal enzyme concerned in the metabolism of GABA, with the use of tetrazolium salts [12].

However, no methods of quantitative evaluation of this reaction in individual structures of the section have yet been worked out. The object of the present investigation was the histochemical study of the enzyme reaction for GABA-T in the presence of different concentrations of substrates and coenzymes and at different temperatures in frozen sections of the rat cerebellar cortex.

EXPERIMENTAL METHODS

Experiments were carried out on 15 male albino rats weighing 150-200 g. The animals were killed by decapitation, the brain was removed, pieces of cerebellar tissue measuring 0.5 × 0.5 cm were cut out and frozen in iso-octane cooled with liquid nitrogen. Histochemical reactions were carried out on frozen sections 10, 15, and 20 μ thick. GABA-T in sections of the cerebellar cortex was detected by Van Gelder's method [12]. The pH was adjusted to 8.4 with 1 N NaOH. Agar was replaced by dextran (mol. wt. 20,000) in a concentration of 60 mg/ml. The effect of α-ketoglutarate (α-ketoglutaric acid, disodium salt; from Boehringer

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Mannheim, West Germany) in a concentration range of $2 \cdot 10^{-4}$-$13 \cdot 10^{-2}$ M, GABA (from Reanal, Hungary) within the range of $2.4 \cdot 10^{-2}$-$9.7 \cdot 10^{-2}$ M, and NAD (from Reanal, Hungary) within the range from $1.2 \cdot 10^{-3}$ to $3 \cdot 10^{-3}$ M, and also of addition of malonic acid in concentrations of 0.5-1 mg/ml, phenazine metasulfate in a concentration of 0.01 mg/ml, and sodium cyanide in a concentration of $1 \cdot 10^{-3}$ M on the velocity of the reaction was studied. The effect of different temperatures (37-43°C) and different thicknesses of the section (10-20 μ) on the kinetics of GABA-T also was investigated. Tetranitroblue tetrazolium (Lachema, Czechoslovakia) was used in all the reactions in a concentration of 0.2 mg/ml.

The reaction was assessed from the rate of increase of optical density in places where formazan was deposited and was expressed as ΔE/min. The optical density was measured on the MUF-5 microcytospectrophotometer at a wavelength of 546 nm with a probe 1 μ in diameter. The effect of changes in the incubation medium on the velocity of the enzyme reaction in the course of time was determined by incubating the sections for 20, 40, and 60 min. The linear segment of the curve of increase of optical density with time was taken as the initial velocity. The experimental results were subjected to statistical analysis [1]. To verify the specificity of the reaction the GABA-T inhibitor aminohydroxyacetic acid (from Sigma, USA) was used in a concentration of 1 mg/ml [13]. To compare the localization of GABA-T and succinate dehydrogenase (SD) activity parallel tests for SH were carried out by the tetrazolium method.

EXPERIMENTAL RESULTS

The distribution of GABA-T activity in sections through the rat cerebellar cortex corresponded in these experiments to the pattern of distribution of the enzyme described previously [6, 11]: The highest activity was found in the Purkinje cells and in structures of the granular layer, whereas moderate and weak activity was found in the molecular layer and white matter respectively (Fig. 1a). Addition of the specific inhibitor, and also removal of any of the substrates led to inhibition of the reaction. Changes in the concentrations of the various ingredients of the incubation medium and in the conditions of incubation (temperature, time) revealed certain characteristic features of the kinetics of GABA-T in the Purkinje cells, granular layer, and white matter. As the graph in Fig. 2a shows, an increase in the α-ketoglutarate concentration between $2 \cdot 10^{-4}$ and $5.2 \cdot 10^{-3}$ M led to an increase in the reaction velocity. A further increase in concentration led to a progressive decrease in the velocity. From the values of the initial reaction velocities at α-ketoglutarate concentrations of $2 \cdot 10^{-4}$-$5.2 \cdot 10^{-3}$ M the value of the Michaelis constant (K_T) of GABA-T for α-ketoglutarate was calculated: For the layer of Purkinje cells and the granular layer its value was $1.7 \cdot 10^{-3}$ M and for the white matter $3.8 \cdot 10^{-3}$ M. A further increase in the α-ketoglutarate concentration gave a curve characteristic of competitive inhibition by an excess of substrate on the double-reciprocal Lineweaver–Burk plot (Fig. 2b). The ability of α-ketoglutarate to inhibit GABA-T competitively has been demonstrated by biochemical experiments [7]. The optimal concentration of α-ketoglutarate giving maximal reaction velocity in the section was thus $5.2 \cdot 10^{-3}$ M (1 mg/ml). Higher concentrations of α-ketoglutarate as used in the experiments of Van Gelder and others [6, 8, 9, 11], detect activity of the enzyme under conditions of partial inhibition that are not suitable for quantitative histochemical investigation of this reaction in sections. A concentration of 1 mg/ml was accordingly used in later experiments.

An increase in the GABA concentration within the range from $2.4 \cdot 10^{-2}$ to $4.9 \cdot 10^{-2}$ M led to a very slight increase in the reaction velocity, but a further increase in the concentration to 9.7 ± 10^{-2} M caused no significant increase in the reaction velocity. A concentration of GABA of $3 \cdot 10^{-2}$ M (3 mg/ml) was accordingly chosen for the subsequent experiments.

The reaction velocity was clearly dependent on temperature, an increase in which from 37 to 43°C led to a twofold increase in the optical density. According to the results of biochemical investigations, the temperature optimum of the purified GABA-T preparation does not exceed 60°C [2].

An increase in the NAD concentration within the range from $1.2 \cdot 10^{-3}$ to $3 \cdot 10^{-3}$ M was accompanied by an increase in the velocity of the enzyme reaction in all structures and an increase in the difference between activity of the Purkinje cell layer and the granular layer; accordingly, in the subsequent experiments a concentration of $3 \cdot 10^{-3}$ M (2 mg/ml) was used, contrary to that recommended by Ritter ($1.2 \cdot 10^{-3}$ M).

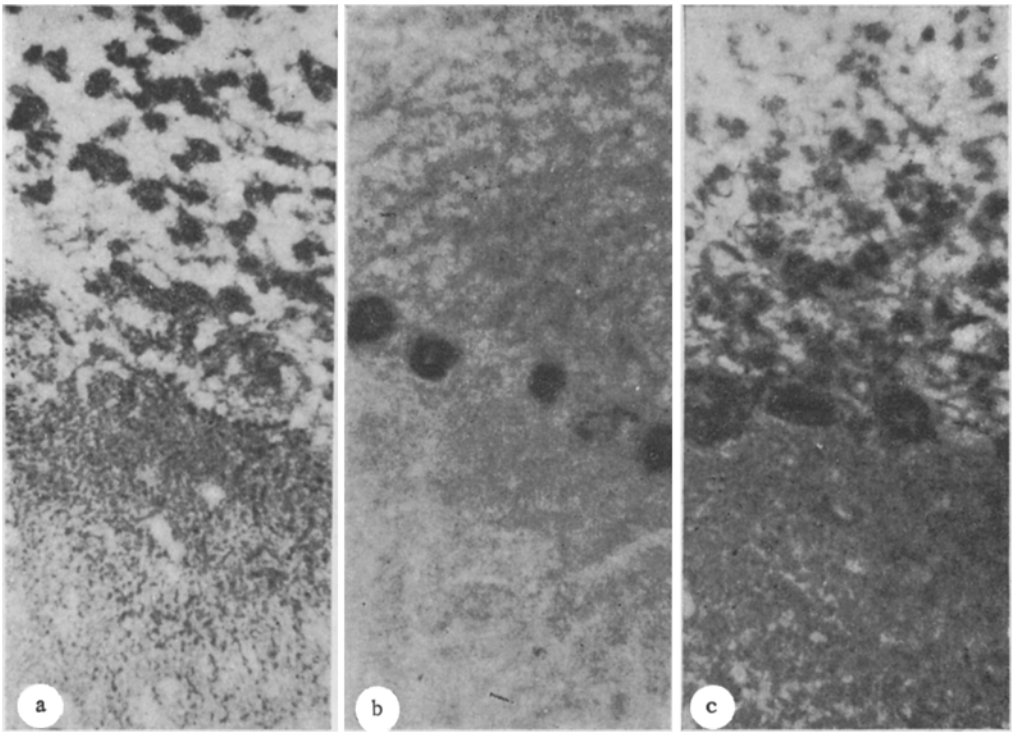

Fig. 1. Distribution of enzyme activity in cerebellar cortex: a) GABA-T; b) GABA-T, incubation with malonic acid; c) SD. 1) Molecular layer; 2) layer of Purkinje cells; 3) granular layer. 400×.

Since reduction of tetrazolium and formazan formation in the histochemical reaction for GABA-T take place through the action of an NAD-dependent enzyme — succinic semialdehyde dehydrogenase — and of NADH-tetrazolium reductase, the effect of phenazine metasulfate (PMS) and of NaCN on the velocity of the enzyme reaction in the sections was investigated. The addition of PMS to the incubation medium considerably increased the reaction velocity on account of rapid nonenzymic transfer of the reduced equivalent from NADH to tetrazolium, bypassing NADH-tetrazolium reductase.

The addition of cyanide increased the velocity of formazan formation chiefly in the structure of the granular layer. Similar results for the effect of PMS and NaCN were obtaine by Ritter [9], who estimated the velocity of the reaction for GABA-T by a method of elution of formazan from the sections.

An additional source of formazan formation in the histochemical reaction for GABA-T is oxidation of succinic acid, formed from succinic semialdehyde. Ritter and Wenk [8] and the present writers showed that the addition of malonate, an inhibitor of SD, to the incubation medium improves the morphological localization of GABA-T (Fig. 1b). Quantitative investigation of the action of malonate within a concentration range of 0.5-1 mg/ml showed a marked inhibitory effect in a concentration of 1 mg/ml. As Fig. 2c shows, this concentration of malonate differed in its effect on the kinetics of the reaction in different layers of the cerebellum. The strongest inhibition was observed in the granular layer, which gave the highest activity in the reaction for SD (Fig. 1c). It was accordingly decided that malonate should be added to the incubation medium for the study of GABA-T activity in structure with a high SD level. Data on the dependence of the reaction velocity on the thickness of the section, given in Fig. 2d, confirm the fact established by many workers that 10 μ is the thickness of section which ensures optimal conditions for the enzyme reaction in the section and gives a good pattern of distribution of enzyme activity in the morphological structures.

On the basis of this investigation the optimal composition of the incubation medium for the detection of GABA-T and for subsequent evaluation of the reaction could be chosen; it differs from the modification used for visual assessment of the reaction.

GABA	3 mg/ml
α-ketoglutarate	1 mg/ml

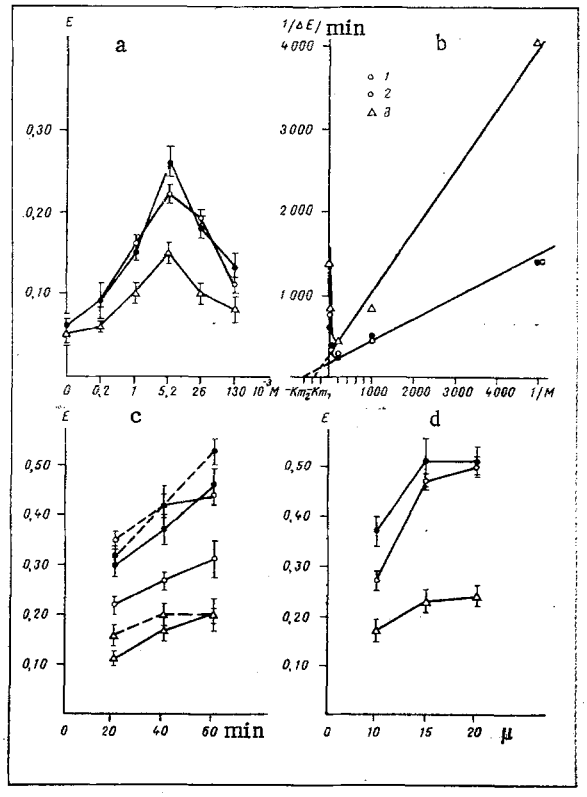

Fig. 2. Effect of composition of incubation medium and thickness of section on course of histochemical reaction: a) effect of α-ketoglutarate concentration on optical density of structures; b) effect of α-ketoglutarate concentration on reaction velocity; c) effect of malonic acid (1 mg/ml) on dynamics of increase in optical density (continuous lines denote incubation with, broken lines without malonic acid); d) effect of thickness of section on optical density of structures. 1) Layer of Purkinje cells; 2) granular layer; 3) white matter.

NAD	2 mg/ml
Tetranitroblue tetrazolium	0.2 mg/ml
Phenazine metasulfate	0.01 mg/ml
Sodium cyanide	0.05 mg/ml
Malonic acid	1 mg/ml

The incubation medium also contained phosphate buffer, with the addition of NaCl, KCl, $CaCl_2$, and $MgSO_4$, as in Van Gelder's formula, and 1 N NaOH to pH 8.4, and also dextran (mol. wt. 20,000) in a concentration of 60 mg/ml. Incubation was carried out at 43°C; the incubation time depended on the original activity of the tissue enzymes and varied from 40 to 60 min.

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