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Method of Assay for 4-Aminobutyrate-2-Oxoglutarate Aminotransferase

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In this paper an analytical colorimetric method is presented where 2,4-dinitrophenyl hydrazine is used to measure small amounts of succinic semialdehyde in the presence of 2-oxoglutarate. This method is applicable to the measurement of 4-aminobutyrate-2-oxoglutarate aminotransferase activity, where succinic semialdehyde formed during the enzymatic reaction has to be measured in the presence of 2-oxoglutarate.

A wide variety of methods have been devised to measure the activity of 4-aminobutyrate-2-oxoglutarate aminotransferase (GABA-T) (14, 16). The two products of the transamination reaction can be measured. Glutamate has been determined colorimetrically and enzymatically, using fluorometric (2) or manometric (1, 8) techniques. Succinic semialdehyde has been measured enzymatically (11) or by conversion to an α-metilated derivative with specific fluorometric properties. PITTS *et al.* (14) described a fluorometric method in which NADH formed during conversion of succinic semialdehyde into succinic acid was measured by means of purified kidney succinic semialdehyde dehydrogenase. JAKOBY *et al.* (12) assayed the GABA-T by measuring the increase in optical density at 340 nm due to the formation of NADPH using purified succinic semialdehyde dehydrogenase from *Pseudomones fluorescens*. A radiometrical method was used by several authors (6, 10, 18) while others (7, 16), used a coupled enzyme method for the assay of GABA-T activity in subcellular fractions.

In the present paper one report a method where the succinic semialdehyde formed during the GABA-T reaction is measured by means of the hydrazone formation from succinic semialdehyde an 2,4-dinitrophenyl hydrazine (2,3-DNPH) under defined conditions. In spite of this variety of methods to measure the GABA-T activity a great number of limitations exist since these methods can not be generally applied; for instances, the radiochemical method is very sensitive but it is not possible to know the effective spe-

cific activity for the substrate and the use of coupling reactions is not very adequate to assay the action of effectors. Our method presents the adventage of measuring directly the succinic semialdehyde in the presence of 2-oxoglutarate.

Materials and Methods

Succinic semialdehyde hydrazone. The method is based on the reaction between succinic semialdehyde and 2,4-DNPH. The semialdehyde hydrazone is extracted with xylol and borate buffer; under these conditions the hydrazone from 2-oxoglutarate is not extracted.

Reagents. 2,4-dinitrophenyl hidrazine at 0.2 % (w/v) in 2 M HCl; Borate buffer pH 10 prepared according to GOMORI (9); Xylol and absolute ethanol; 5 M NaOH; 10 % trichloroacetic acid (w/v) prepared extemporaneously; Succinic semialdehyde prepared and purified according to BRUCE *et al.* (5) and evaluated by the hydrazone method (4) using pyruvate as standard.

Succinic semialdehyde assay. Samples with different concentrations of succinic semialdehyde in a volume of 200 μl were kept in an ice-bath and the following procedures carried out:

Add 300 μl of 10 % trichloroacetic acid; Add 1 ml of 2,4-DNPH at intervals of 30 s and keep in the ice-bath for 8 min exactly; Add 5 ml of xylol and stir mechanically for 30 s; Keep at room temperature until separation of two layers; Take 4 ml of the uper layer and add 3 ml of borate buffer, stir mechanically during 30 s Keep at room temperature until separation of the two layers and remove the upper layer; Take 2 ml from the lower layer and add 2 ml of absolute ethanol; Keep for 5 min and then add 1 ml of 5 M NaOH; Measure the absorbance at 420 nm in less than 3 min after addition of NaOH.

Enzyme preparation. Rat brains, Wistar strain, were homogenized in mechanical glass pestle Potter, with 6 volumes of 0.3 M Tris-HCl buffer pH 8.6 containing 0.5 % Triton X-100 and then centrifuged at 0° C for 30 min at 35.000 × *g*. The supernatant was used as enzymatic preparation.

Proteins were measured by LOWRY *et al.* method (13).

Results

The formation of hydrazones from succinic semialdehyde at the experimental conditions was lineal versus succinic semialdehyde, at least, between concentrations of 0.09 to 0.74 μmoles of succinic semialdehyde. The best resolution was obtained when 10 mM of 2,4-DNPH (5 mM in the reaction medium), were used (fig. 1). Higher 2,4-DNPH concentrations were not used because the low solubility of this reagent in aquous solutions. Under the experimental conditions described above, the hydrazones from 2-oxoglutarate were not extracted. The presence of 2-oxoglutarate in the reaction medium diminished the sensibility in the formation of hydrazone from succinic

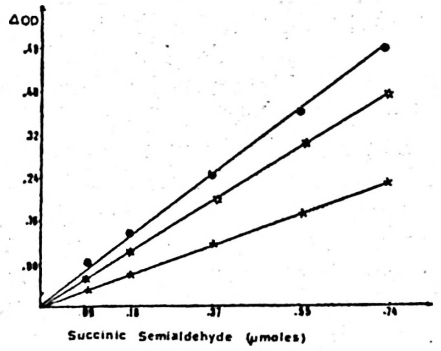

Fig. 1. *Effect of 2,4-DNPH on formation of hydrazones from succinic semialdehyde.* Succinic semialdehyde at indicate concentrations, in a total volume of 1 ml plus 1 ml of 2,4-DNPH concentration of *—*—* 1 mM; ✻–✻–✻ 5 mM and ●–●–● 10 mM.

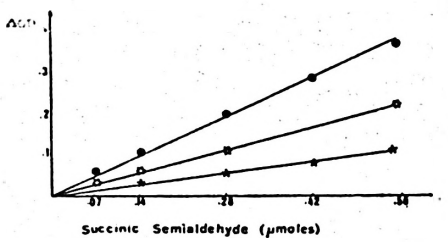

Fig. 2. *Effect of 2-oxoglutarate on formation of hydrazones from succinic semialdehyde.* Succinic semialdehyde at the indicate concentrations in a total volume of 200 μl plus: ●—●—● without 2-oxoglutarate; ✻—✻—✻ with 10 mM of 2-oxoglutarate; ✻—✻—✻ with 25 mM of 2-oxoglutarate. The hydrazones from succinic semialdehyde were checked as indicated in Materials and Methods.

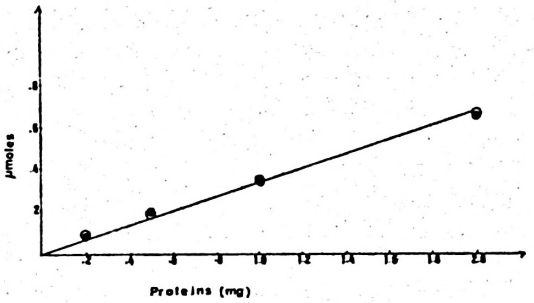

Fig. 4. *Effect of the enzyme concentration on GABA-T activity using two concentrations of 2-oxoglutarate.* As in figure 3 except that the concentration of 2-oxoglutarate was: ●—●—● 12.5 mM or O—O—O 31 mM, and the protein concentration as indicated.

semialdehyde but without loss of lineality (fig. 2) what allowed the evaluation of of the real concentration of succinic semialdehyde present in the medium.

Experiments with rat brain homogenates were carried out to establish the optimal conditions for the assay of GABA-T activity. The rate of GABA-T from whole brain homogenates was lineal versus both, incubation time (fig. 3) and enzyme concentration (fig. 4).

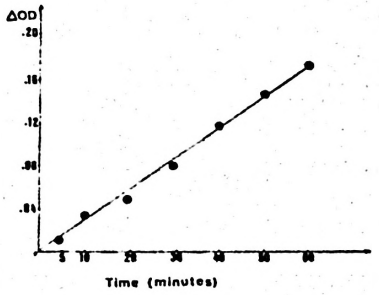

Fig. 3. *Effect of the Incubation time on GABA-T activity when the reaction is measured by the hydrazone method.* 30 μmoles Tris-HCl buffer pH 8.6; 12.5 μmoles of 2-oxoglutarate; 12.5 μmoles of GABA; 0.008 μmoles of reduced glutation; 0.016 μmoles of PLP, about 1 mg protein (from enzymatic extract) in a total volume of 200 μl. Incubations were carried out during 1 hour. The reaction was stopped by addition of 300 μl of 10 % trichloroacetic acid. The supernatant, after centrifugation, was used to measure the succinic semialdehyde formed during reaction.

The enzymatic activity was checked at two 2-oxoglutarate concentrations, namely 31 and 12.5 mM; in both cases the reaction was lineal. The enzymatic activity, when 31 mM of 2-oxoglutarate was used was not different from that with 12.5 mM (fig. 4) which means the absence of interferences of 2-oxoglutarate in the assay of the succinic semialdehyde formed during the reaction.

A standard curve of succinic semialdehyde in the presence of the adequated 2-oxoglutarate concentrations (fig. 2) was obtained for each set of samples in order to allow correlation of the optical density and μmoles of succinic semialdehyde present.

Discussion

It is described an analytical method based on the use of 2,4-DNPH to determine succinic semialdehyde in the presence of 2-oxoglutarate, which is applica-

ble in the valoration of GABA-T activity where hydrazones from succinic semialdehyde are assayed in the presence of 2-oxoglutarate. Although 2-oxoglutarate interferes with the succinic semialdehyde hydrazone formation (fig. 2) it is possible to calculate the real formation of succinic semialdehyde hydrazones. It must be taken into account that the sensibility of this method is a great advantage since the reaction could be measured even at low enzyme concentration while references in the literature require higher concentrations (18). This method shows several advantages: 1) Over spectrophotometric methods because there are coupled methods and require a great additional work because succinic semialdehyde dehydrogenase need to be purified from *Pseudomones fluorescens*. 2) Over radiometrically methods because we can not measured the enzyme activity with higher concentrations of substrates which insure the measure of the activity in optimum conditions; and 3) because with our method several samples can be simultaneously assayed.

Resumen

Se presenta un método colorimétrico utilizando 2,4-dinitrofenilhidrazina, mediante el cual pueden medirse pequeñas cantidades de semialdehído succínico en presencia de 2-oxoglutarato. Es aplicable en la valoración de la 4-aminobutirato-2-oxoglutarato aminotransferasa (GABA-T), en donde el semialdehído succínico formado durante la reacción enzimática tiene que medirse en presencia de 2-oxoglutarato, uno de los sustratos de la reacción.

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