DETERMINATION OF PHENYLALANINE HYDROXYLASE ACTIVITY IN LIVER TISSUE

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A micromethod for determination of liver phenylalanine-4-hydroxylase activity has been developed which is suitable for estimating the activity of the enzyme in a few milligrams of tissue obtained at biopsy. The activity of this enzyme has been determined in liver tissue from various animals, from healthy adult humans, and from children with phenylpyruvate oligophrenia.

An important role in the conversions of phenylalanine in mammals is played by phenylalanine-4-hydroxylase (1.14.3.1), an enzyme catalyzing hydroxylation of the aromatic ring. This enzyme is found in the liver in the endoplasmic reticulum fraction. The increased attention paid to determination of this enzyme in recent years is explained by its pathogenetic role in phenylpyruvate oligophrenia, a very severe and relatively widespread hereditary disease.

The direct determination of the activity of this enzyme in liver tissue obtained at biopsy is interesting both for the study of the pathogenesis and for the more detailed diagnosis of this disease. Only macromethods for determination of phenylalanine-4-hydroxylase activity are described in the literature, and they are not suitable for work with the very small amount of tissue yielded by aspiration biopsy. The most widely used method of determining the activity of this enzyme is that based on determination of the tyrosine formed during the reaction, with the aid of nitroso-naphthol reagent [3, 4]. Despite its specificity, this method is not sufficiently sensitive and it is very time consuming [5].

The writers have used a more sensitive method for determination of tyrosine, based on Folin's reaction, and have developed an ultramicromethod for determining the activity of the enzyme which can be used to study it in the few milligrams of tissue obtained by needle biopsy.

EXPERIMETAL METHOD

Reagents: 1) 0.14 M KCl containing 0.0025 M NaOH (pH 7.4); 2) 1 M phosphate buffer (pH 6.7), which is diluted 1 : 2 immediately before the determination; 3) a 0.025 M solution of nicotinamide in 0.5 M phosphate buffer, pH 6.7 (3 mg nicotinamide is dissolved in 1 ml buffer); 4) 0.01 M L-phenylalanine; 5) 0.003 M solution of NAD · H_2; 6) 20% solution of TCA; 7) 2% solution of sodium carbonate in a 0.1 N solution of caustic soda – solution S; 8) Folin's reagent, diluted 1 : 2 before the determination; full details of the method of preparation of Folin's reagent are given in [1].

A calibration curve is drawn for a standard solution of tyrosine containing 5 μmoles/ml or 0.9 mg L-tyrosine per ml.

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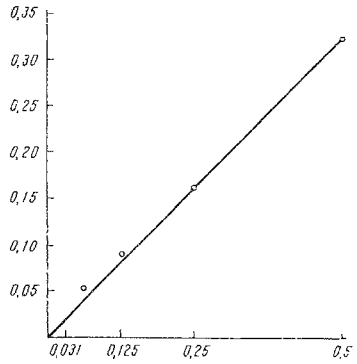

Fig. 1. Calibration curve plotted for a standard solution of L-tyrosine. Abscissa, tyrosine concentration (in μmoles/ml); ordinate, optical density.

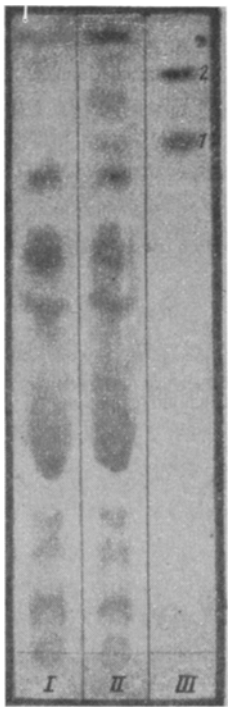

Fig. 2. Chromatography of protein-free supernatant from liver tissue of experimental and control samples. I) Control sample; II) experimental; III) witnesses: 1) tyrosine; 2) phenylalanine.

Course of determination: 25 mg liver tissue is triturated for 30 sec in the cold in a Potter's homogenizer, after which 0.125 ml of cold 0.14 M KCl solution is added and the homogenization continued for a further 30 sec. The homogenate is transferred to a syringe and passed through two layers of Kapron into a cooled centrifuge tube. Centrifugation is carried out for 45 min at 16,000 g and at between −2 and +3°C. The supernatant is used as the source of enzyme.

Incubation is carried out in polycentrifuge tubes, which are fitted into holes in a swimming pool reactor. By means of semiautomatic pipets 0.02 ml of nicotinamide solution in 0.5 M phosphate buffer and 0.02 ml of NAD · H_2 solution are added to the tubes. The reactor is placed in a water thermostat at 38°. After 15 min 0.06 ml supernatant is added to the tubes in the reactor and the contents are mixed and heated for 1 min at the same temperature. Next, 0.02 ml of 0.01 M phenylalanine solution is added and incubation continued for a further 15 min. The reaction is stopped by the addition of 0.06 ml of 20% TCA, after which 0.06 ml distilled water is added; 10 min later the contents of the tubes are centrifuged for 10 min at 5000 rpm.

Simultaneous control tests are carried out to determine the amount of tyrosine preformed in the samples. For this purpose, 0.02 ml of nicotinamide solution in 0.5 M phosphate buffer, 0.02 ml NAD · H_2 solution, 0.06 ml supernatant, 0.02 ml of 0.01 M solution of L-phenylalanine, and 0.06 ml of 20% TCA solution are introduced into the receivers of a comparator. The control samples are incubated for 15 min, after which 0.06 ml distilled water is added, and 10 min later the contents are centrifuged at 5000 rpm for 10 min.

The protein-free supernatant is transferred in a volume of 0.04 ml into microtubes, 0.2 ml of solution S is added, and the contents are thoroughly stirred. Ten minutes later, 0.02 ml of twice diluted Folin's solution is added to each tube. The contents of the tubes are mixed, and 30 min later the samples were examined colorimetrically with the FEK-N-57 instrument at 750 mμ (No. 8 filter) against distilled water with the aid of microcuvettes.

Before the enzyme activity is calculated, the extinction of the control sample is subtracted from the extinction of the experimental sample:

$$E_{exp} - E_{con} = E_{enz},$$

where E_{enz} represents the extinction corresponding to the enzymic activity of the tested solution, E_{exp} the extinction of the experimental sample, and E_{con} the extinction of the control sample.

The extinction value reflecting phenylalanine hydroxylase activity thus obtained is compared with the calibration curve, to give the amount of tyrosine (in micromoles) formed in the sample in 15 min (a).

Activity of the enzyme is calculated by the formula:

$$A_{enz} = 4ab,$$

where A_{enz} represents the activity of phenylalanine-4-hydroxylase expressed as the number of micromoles tyrosine formed from phenylalanine during incubation of 1 g tissue for 1 h; a is the amount of tyrosine found from the calibration curve; and b is the total dilution of the homogenate.

It will be clear from an examination of the graph of optical density as a function of tyrosine contents (Fig. 1) that this method can be used to determine very small amounts of tyrosine: less than 0.06 μmole tyrosine per ml or 0.0024 μmole tyrosine per sample.

TABLE 1. Phenylalanine-4-hydroxylase Activity in Liver of Various Animals and Man

Test object	Phenylalanine hydroxylase activity (in μmoles tyrosine/g liver tissue/h)
Rats	24.9±1.8
Mice	4.91±0.57
Guinea pigs	2.26±0.22
Rabbits	1.83±0.16
Healthy adults	1.4±0.32
Children with oligophrenia	0.0

In the course of development of the micromethod for determination of phenylalanine hydroxylase activity, comparative estimations were made of the activity of the enzyme in rat liver using the suggested micromethod and paper chromatography. For this purpose, 40 μl of the protein-free supernatant from the experimental and control samples used for the determination of tyrosine with Folin's reagent was applied to chromatography paper, and chromatography was carried out in a butanol – acetic acid – water system. Tyrosine was estimated quantitatively by a micromethod developed earlier [2]. The results of the determination of tyrosine by the two methods were very close (Fig. 2). It must be pointed out that the values for the content of preformed tyrosine obtained by the use of Folin's reagent were considerably higher than the values obtained by chromatography, confirming reports in the literature of the inadequate specificity of Folin's method [5].

The sensitivity of Folin's method and of the method of estimation of tyrosine by means of ninhydrin differs only slightly, according to the writers' findings. The smallest quantity of tyrosine which can be determined in a sample by the use of micromethod with Folin's reagent is 0.0024 μmole (Fig. 1). The smallest quantity of tyrosine which can be determined in the form of the copper derivative with ninhydrin is also 0.002 μmole per sample, i.e., 0.36 μg [2].

The writers have used this micromethod for determination of liver phenylalanine-4-hydroxylase activity in order to investigate the activity of this enzyme in the liver tissues of various animals, of healthy adult persons, and of children with phenylpyruvate oligophrenia (Table 1).

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