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Liver Phenylalanine Hydroxylase Assay

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PRELIMINARY ASSAY

The enzymatic hydroxylation of phenylalanine to tyrosine is complex and the overall reaction consists of two component steps:

$$\text{phenylalanine} + \text{tetrahydropteridine} + O_2 \rightarrow \text{tyrosine} + \text{"quinoid" dihydropteridine}$$

$$\text{"quinoid" dihydropteridine} + \text{NADPH} + H^+ \rightarrow \text{tetrahydropteridine} + NADP^+$$

$$\text{phenylalanine} + \text{NADPH} + H^+ \xrightarrow{+O_2} \text{tyrosine} + NADP^+ + H_2O$$

The first reaction is catalyzed by phenylalanine hydroxylase and the second reaction, which generates the reduced form of pteridine cofactor (biopterin), is catalyzed by dihydropteridine reductase (1–3). A direct assay of phenylalanine hydroxylase can be achieved by supplying optimal concentrations of reduced pteridine cofactor or an analog of the reduced cofactor (6,7-dimethyl-5,6,7,8-tetrahydropterine) maintained in its reduced state by the addition of NADH or NADPH (4). The assay avoids the need for an accessory NADPH enzyme-generating system and ensures that the only rate-determining component in the hydroxylating reaction is the concentration of phenylalanine hydroxylase.

A typical assay for phenylalanine hydroxylase is given in Table 1. The control flasks contain all of the reagents except L-phenylalanine. Incubations are carried out at 37° in a 10-ml Erlenmeyer flask in a Dubnoff shaker, in air, and the reaction is started by the addition of enzyme to the control and experimental flasks. The shaking rate is 140 oscillations per minute. The rate of the reaction was linear with time for at least 30 min and is proportional to enzyme concentration. After 8 min of incubation the reaction mixtures are deproteinized with 0.1 ml of 20% trichloroacetic acid, to stop the reaction, and placed in ice for 5 min. The acidified contents of the flasks are centrifuged at 3000*g* for 5 min and 0.5 ml of the

TABLE I
PHENYLALANINE HYDROXYLASE ASSAY

	Amount added: 0.05 M sodium phosphate buffer, pH 7.0, containing 0.005% mercaptoethanol (ml)	NADH (0.0014 M)/ $DMPH_4$ (0.0007 M) (ml)	Liver homogenate (2.5%, w/v) (ml)	L-Phenylalanine (0.002 M) (ml)
Control flask	0.4	0.2	0.1	—
Experimental $flask_1$	0.25	0.2	0.1	0.15
Experimental $flask_2$	0.25	0.2	0.1	0.15

supernatant fraction is used to determine the amount of tyrosine formed by the colorimetric method, based on the chromagen formed with α-nitroso-β-naphthol and tyrosine (5, 6). Under the conditions of the assay, 1.0 μg of tyrosine formed gave an optical density unit of 0.051 at 450 nm.

Reagents

L-phenylalanine was used, 15.42 mg per 10 ml of 0.05 M sodium phosphate buffer, pH 7.0. A mixture of 3.17 mg of $DMPH_4$ and 17.38 mg of $NADH \cdot Na_2$ was dissolved in 5 ml of 0.05 M sodium phosphate, pH 7.0; it was freshly prepared prior to use and kept in ice.

LIVER HOMOGENATE PREPARATION

Small samples of liver (10 to 50 mg) are prepared as a 2.5 or 5% (w/v) whole homogenate in 1.15% KCl, 0.005% mercaptoethanol, pH 7.0, or 0.05 M sodium phosphate buffer, 0.005% mercaptoethanol, pH 7.0, with a Potter–Elvehjem glass homogenizer, at 5°. The homogenate is centrifuged at 10,000*g* for 10 min and the supernatant fraction is used for the phenylalanine hydroxylase assay. Mouse, rat, guinea pig, and human autopsy homogenates were stable from 7 to 9 days when frozen. Human autopsy homogenates lost on the order of 15% hydroxylase activity after 6 days of freezing. Small samples of mouse, guinea pig, and human autopsy liver samples (20–30 mg) were wrapped in Saran wrap (to minimize dehydration) and frozen immediately (dry ice); these maintained their initial activity for at least 3 days. Simulated autopsy sampling of guinea pig liver (whole animal kept at 5°) at various times indicated no significant loss of phenylalanine hydroxylase activity for at least 8 hr; samples taken at 24 hr lost 30–40% of the initial activity.

TABLE 2
REAGENTS AND AMOUNTS FOR COLORIMETRIC DETERMINATION OF L-TYROSINE

	Acidified supernatant	L-Tyrosine (ml)	α-Nitroso-β-naphthol (ml)	$NaNO_2$ (ml)
Control flask	0.5 ml	—	0.25	0.25
Experimental flask$_1$	0.5 ml	—	0.25	0.25
Experimental flask$_2$	0.5 ml	—	0.25	0.25
Blank	0.5 ml H_2O	—	0.25	0.25
Standard tyrosine (2 μg)	0.3 ml H_2O	0.2	0.25	0.25
Standard tyrosine (2 μg)	0.3 ml H_2O	0.2	0.25	0.25

Under the conditions of the assay, the reaction rate was linear with time for at least 30 min, proportional to liver homogenate concentration, and the reaction could be performed at 27 or 37°. The temperature coefficient was found to be 2.07; the apparent K_m of phenylalanine hydroxylase in human autopsy liver samples was found to be $1.2 \times 10^{+3}$ M for phenylalanine and 0.66×10^{-4} M for $DMPH_4$.

α-NITROSO-β-NAPHTHOL COLORIMETRIC DETERMINATION OF L-TYROSINE

The reagents are pipetted into glass-stoppered conical tubes (15 ml capacity) and incubated for 30 min at 55°, allowed to cool to room temperature, and then are extracted with 5.0 ml of ethylenedichloride. An aliquot of the aqueous layer is read at 450 nm. The chromagen is stable for at least 30 min.

Reagents

α-Nitroso-β-naphthol, 50 mg per 25 ml of 95% ethanol (stable for at least 1 week at 5°); freshly prepared sodium nitrite, 25 mg/25 ml of 20% HNO_3; and freshly prepared L-tyrosine, 5.0 mg per 500 ml of 0.01 M sodium phosphate buffer, pH 7.0, heated to 60° to dissolve, were used.

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