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Determination of Plasma and Tissue Levels of Tyramine by Radioimmunoassay[1,2] (38875)

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Tyramine (TYR) is a naturally occurring sympathomimetic amine which has been identified as a normal constituent of mammalian tissues (1). A major interest in TYR is in its pharmacological effects on the cardiovascular system, presumably occurring through the release of norepinephrine from endogenous stores in tissues (2). Recently it has been shown that in about 30% of patients with classical migraine, headaches can be provoked by TYR and TYR-containing foods such as cheese (3–5). Patients treated with monoamine oxidase inhibitors have been known to experience severe and occasionally fatal hypertensive attacks after eating cheese with high TYR content (6). Elevated levels of TYR may occur under such circumstances and in hypertyrosinemia of premature babies (7–9).

The conventional assay of TYR in biological fluids and food products has depended on time-consuming extraction procedures coupled with fluorometric analysis (1, 10–12). A number of workers have investigated gas–liquid chromatographic (glc) methods for quantifying TYR (13, 14). While these procedures are promising, problems inherent in preparing biological samples for glc and interfering side reactions in the derivatization of TYR tend to make this alternative less attractive.

We considered the possibility of using a radioimmunoassay for the analysis of TYR; this technique, which is based on the competition between labeled and unlabeled hapten for binding to a limited number of sites on a specific antibody, has been employed to quantitate biologically important molecules in picogram and nanogram quantities even in unprocessed serum or urine (15, 16). We report, herewith, the development of a sensitive and specific radioimmunoassay for the quantitative determination of TYR. This assay was utilized to measure in rabbits: (a) the plasma level half-life of administered TYR and (b) endogenous tissue levels of TYR.

Materials and Methods. Tyramine, 3-*O*-methyldopamine, phenethylamine, L-amphetamine, *p*-hydroxynorephedrine, serotonin, *p*-hydroxyphenylacetic acid, and L-tyrosine were obtained from Aldrich Chemical Company. Dopamine, L-norepinephrine, L-epinephrine, and methylated bovine serum albumin (mBSA) were obtained from Sigma Chemical Company. 3,4-Dimethoxyphenethylamine was obtained from Nutritional Biochemical Corporation. *p*-Methoxyphenethylamine and *p*-hydroxyphenethanol were synthesized in our laboratories, according to a published procedure (17). *p*-Acetoxyphenethylamine, *m*-tyramine and *N*-methyltyramine were supplied to us by Dr. R. J. Borgman, West Virginia University, and *p*-hydroxyamphetamine was donated by Smith Kline and French Laboratories. *p*-Aminohippuric acid (PAH) was obtained from Eastman Chemical Company and 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide from Ott Chemical Company (stored at −10°). Tyramine-^{3}H (sp act 6.7 Ci/mmole) and tyramine-^{14}C (sp act 12.4 mCi/mmole) were obtained from New England Nuclear. Freund's complete adjuvant and human gamma globulin were obtained from Miles Laboratories. Rabbits

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(males, New Zealand strain) were purchased from Hiram Davis, Stockbridge, GA and caged individually. These were maintained on Purina Rabbit Chow and water ad lib.

Preparation of antigen. To a solution of 50 mg of PAH in sodium acetate buffer (5 ml, 0.05 *M*, pH 6) was added 50 mg of mBSA and 70 mg of 1-ethyl-3-(3′-dimethylaminopropyl) carbodiimide at room temperature. The mixture was stirred at 4° for 24 hr and then dialyzed for 6 days against Sörenson phosphate buffer (M/15; pH 7.6). The PAH–mBSA conjugate solution was adjusted to pH 1 with 1 *N* HCl, cooled (0°), and to it was added dropwise a solution of 100 mg of $NaNO_2$ in 1 ml of water. The diazotized protein solution was then added dropwise to TYR·HCl (100 mg) in 0.5 *N* NaOH (5 ml) with stirring at 0° (Fig. 1). An orange color developed immediately; the reaction mixture was stirred overnight at 4° and then dialyzed for 5 days against distilled water with three changes (2 liters) per day. The extent of conjugation of TYR to the protein was estimated by carrying out the reaction with TYR-^{3}H (2 μCi). The number of TYR residues coupled per mole of mBSA was determined by a column technique and by measuring protein and ^{3}H concentrations in the antigen solution (18). The antigen solution was lyophilized and stored at −20°.

Immunization procedure. Rabbits were immunized with antigen (5 mg; approx 100 μg of TYR equivalent). The immunogen was dissolved in 50% aqueous dimethylsulfoxide and emulsified with an equal volume of Freund's complete adjuvant. The initial dose was 0.25 ml per footpad. A booster injection of 2.5 mg of the antigen in adjuvant was given subcutaneously in the dorsal region of the neck every 3 wk. Blood was collected 7–10 days after booster injection and antiserum analyzed for antibodies to TYR. Control serum was collected from untreated rabbits.

Detection of antibodies. The immunological potency of TYR antibody was determined by a modification of the method of Mu *et al.* (18). Into 12 × 75-mm plastic tubes were placed (Lab-Tek culture tube, Scientific Products, Inc.) 0.7 ml of a solution of 0.2% human gamma globulin w/v

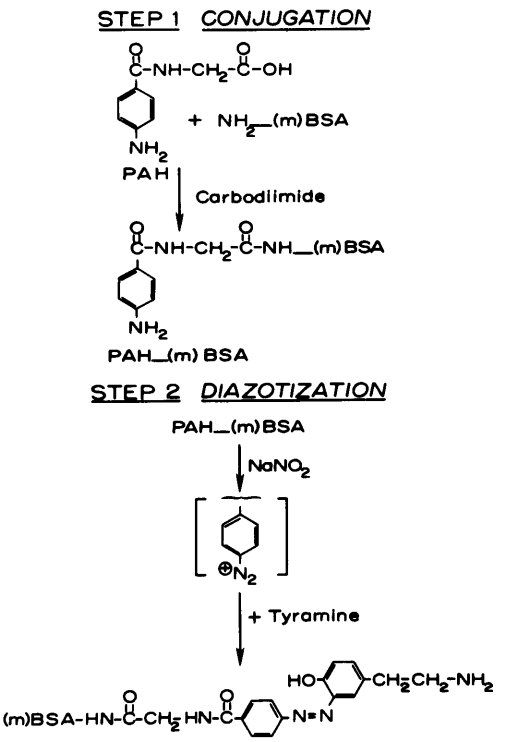

FIG. 1. Synthesis of TYR antigen. Diazotization was carried out at pH 1.

in phosphate buffer (pH 7.6, 0.01 *M* sodium phosphate containing 0.15 *M* NaCl and 0.1% Na azide w/v). Then 0.1 ml of 0.1 *M* Na_2 EDTA (pH 7.6), 0.1 ml of various dilutions of antiserum, and 0.16 ng of tyramine-^{3}H (7500 cpm) in 0.1 ml of buffered saline were added. The tubes were capped and incubated at 0–4° for 24 hr. After incubation, the free and antibody-bound TYR were separated by ultrafiltration. Dialysis tubing (U-shaped; cellulose, ¼ in., size 8, VWR Scientific, Inc.) was placed into a plastic Lab-Tek culture tube (12 × 75 mm) in such a way that about ¼ in. protruded on each side. Essentially all of the incubation solution was transferred into the dialysis tubing. The tubes were capped and centrifuged at 2000*g* at 4° for 2 hr; 0.15 to 0.2 ml of ultrafiltrate was formed. Aliquots (0.1 ml) of both inside and outside phases were dissolved in 18 ml of scintillation fluid (prepared from 7 g PPO, 0.36 g POPOP, 200 ml Beckman Biosolv BBS-3, and 1 liter toluene) and ^{3}H was measured in a Beckman (LS-133) liquid scintillation spectrometer (counting

efficiency = 40% and 88% for ^{3}H and ^{14}C, respectively). Each control and unknown sample was assayed in triplicate. All samples were counted to ±2% error.

Radioimmunoassay procedure. The assay was carried out in a fashion similar to the above procedure. Into Lab-Tek culture tube was placed 0.6 ml of 0.2% human gamma globulin in phosphate-buffered saline and then 0.1 ml of Na_2 EDTA (0.1 *M*), 0.1 ml of antiserum (final dilution 1:50), and 0.1 ml of unlabeled TYR (0.5–50 ng). The mixture was incubated overnight (4°). At this point 0.1 ml of TYR-^{3}H (0.16 ng, 7500 cpm) in buffer was added and the incubation continued for 24 hr. The free and antibody-bound TYR-^{3}H fractions were separated by ultrafiltration. Appropriate blanks containing normal rabbit serum instead of antiserum were included in each assay. Normal rabbit serum did not bind TYR-^{3}H.

Measurement of plasma levels and tissue distribution of tyramine. Rabbits (2.5 kg) were anesthetized with sodium pentobarbital (30 mg/kg iv). Catheters were inserted into a femoral vein for TYR administration and a femoral artery for blood sampling. Rabbits were given 0.6 μmole/kg of TYR-^{14}C (10 μCi). Blood was collected at various time intervals up to 120 min. The plasma was obtained by centrifugation (500*g*) and analyzed immediately for TYR by two independent methods: (a) radioimmunoassay and (b) extraction with ethyl acetate followed by thin-layer chromatography and counting according to the methods of Oates (10) and Wall (19). In these experiments, standard curves were obtained with control plasma to which appropriate amounts of TYR had been added using exactly the same procedure. In order to determine the endogenous tissue levels of TYR, rabbits were sacrificed by ether inhalation, selected tissues were removed, weighed, cooled (0°), and then homogenized (20% w/v) in 0.1 *N* HCl. The homogenates were centrifuged at 10,000*g* for 20 min at 4°. The supernatant was removed and stored immediately at −80° until analyzed by radioimmunoassay (within 24 hr). In preliminary studies 0.5–50 ng of TYR was added to brain homogenate (5% w/v) and radioimmunoassay was carried out. Comparisons were made with standard curves of TYR in buffers.

Measurement of tyramine levels in urine. Tyramine was determined in the urine of rabbits both by ^{14}C measurement and radioimmunoassay by procedures described for plasma. Urine was collected for 0–1 hr after administration of TYR-^{14}C. A sample of blank urine was obtained prior to dosing.

Results. Tyramine, like other low-molecular-weight molecules (20), was linked to albumin to render it antigenic. Tyramine was conjugated to mBSA with a coupling ratio of 25 moles of TYR/mole of mBSA (assuming a mol wt of 70,000).

Antibodies against TYR could be detected in rabbits 8 wk after immunization. There was a significant increase in binding of TYR-^{3}H when compared to control serum taken from the same rabbit before immunization or serum from other nonimmunized rabbits. After 4 mo of immunization a final dilution of 1:50 of the antiserum could bind 50% of the TYR-^{3}H. The sensitivity of the assay is illustrated in Fig. 2. About 1 ng of TYR can be detected with the antiserum. The sensitivity of the assay was improved 5-fold by initially incubating unlabeled TYR with the antibody, and then adding labeled TYR. The present result is in agreement with a previous observation of improvement of assay sensitivity by delayed addition of labeled hapten (21). When TYR was added to plasma and brain homogenate and the radioimmunoassay was carried out, the recovery of TYR from these biological fluids

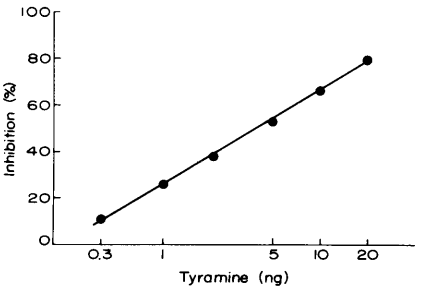

FIG. 2. A typical standard curve of the inhibition of the binding of TYR-^{3}H by antiserum and TYR-PAH-mBSA complex by nonradioactive TYR sample in 0.01 *M* phosphate-buffered saline. Each point represents the average of three determinations.

TABLE I. INHIBITION OF BINDING OF TYR-^{3}H BY ANTI-TYR SERUM BY VARIOUS TYR ANALOGS AND METABOLITES.

Compound[a]	I_{50}[b] (ng)
Tyramine	4
p-Acetoxyphenethylamine	50
p-Methoxyphenethylamine	450
Dopamine	170
3-*O*-Methyldopamine	165

[a] The following compounds required >500 ng to produce 50% inhibition: *m*-tyramine, *N*-methyltyramine, phenethylamine, L-norepinephrine, L-epinephrine, L-amphetamine, *p*-hydroxyamphetamine, *p*-hydroxynorephedrine, serotonin, 3,4-dimethoxyphenethylamine, octopamine, *p*-hydroxyphenylacetic acid, *p*-hydroxyphenethanol, *p*-hydroxymandelic acid, L-tyrosine and L-dopa.

[b] Nanograms added to 1 ml incubation mixture to cause a 50% inhibition of binding of TYR-^{3}H to anti-TYR serum.

was essentially quantitative (90–95%). This indicated the absence in these samples of substances capable of interfering in the recovery of TYR. The anti-TYR serum when stored at −80° showed no significant change in activity for 6 mo.

The specificity of the antibody was tested with several metabolites, and analogs of TYR; the results are shown in Table I.

The radioimmunoassay described above was used to measure TYR in plasma and urine of rabbits after iv administration of 0.6 μmole/kg of TYR-^{14}C. The data revealed a rapid decline in plasma TYR levels during the first 10 min ($t_{1/2}$ 2 min) after administration of TYR-^{14}C followed by a much slower decline ($t_{1/2}$ 54 min) as shown in Fig. 3. A comparison of the radioimmunoassay procedure with a radiochromatographic method indicated good agreement for plasma TYR levels (Fig. 3). Study of the distribution of TYR in tissues of rabbits revealed a consistent high localization of TYR in adrenals and spleen (Table II). Analysis of the urine samples for TYR levels by these methods also indicated good agreement. In a typical experiment the TYR levels by the two methods (radioimmunoassay and radiochemical) were 909 and 980 ng/ml, respectively. For the blank urine the value was 35 ng/ml.

Discussion. A radioimmunoassay procedure is described for the measurement of TYR which has the attributes of being simple, sensitive, and highly specific. The radioimmunoassay of TYR permits its estimation at nanogram levels in tissue extracts and biological fluids.

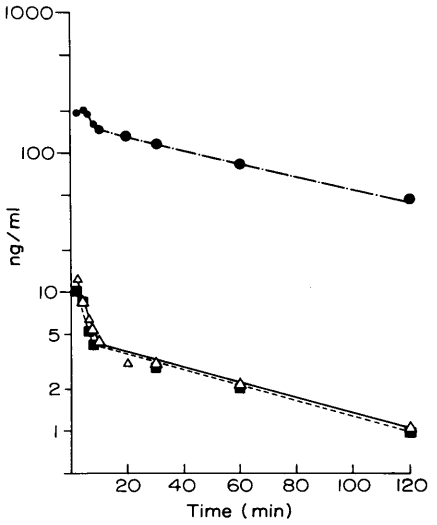

FIG. 3. Average plasma levels of TYR after iv administration of 0.6 μmole/kg of TYR to rabbits. Comparison of analysis of TYR by specific tracer experiments (△——△) and radioimmunoassay (■——■). Plasma levels of ^{14}C (●-·-·-·-●) given as ng/ml TYR equivalent. Each point represents the average for five animals. The range of concentrations (ng/ml) for the 2, 4, 6, 8, 10, 20, 30, 60, and 120 min were (12–13, 8–9, 6–7, 5–6, 4–5, 2–4, 2–4, 1.5–2.7, 1–1.3), and (8–12, 5–12.4, 4.5–5.3, 3.5–5, 3.2–4.8, 2.3–3.4, 1.5–2.1, 1–1.6) for the levels of TYR in rabbit plasma determined by tracer experiments and radioimmunoassay, respectively. For total ^{14}C given as ng/ml TYR equivalent the levels were (189.5–195.4, 202.4–210, 190–192, 159.91–163.45, 149.23–151.72, 140.12–142.69, 75.78–78.73, 43.12–44.14). The average volume of distribution of TYR is 16 liters/kg.

The production of specific antisera is influenced by various factors such as differences in bridge length between hapten and carrier, degrees of freedom, and site of conjugation (22–24). It is of interest that the insertion of PAH as a bridge between mBSA and TYR (without the loss of functional groups of the latter), resulted in the formation of antibodies that were specific both to the ring and the side-chain moiety of the TYR molecule (Fig. 1, Table I).

A number of analogs of TYR were em-

TABLE II. TISSUE DISTRIBUTION OF ENDOGENOUS TYR IN RABBITS[a]

Tissue	Concentration of TYR (ng/g)	Tissue/plasma concentration ratio
Heart	110.0 (88.0–132.0)	44.0
Adrenal	656.0 (511.0–766.0)	262.4
Spleen	512.5 (400.0–600.0)	205.0
Brain	185.7 (141.0–212.0)	74.0
Lung	183.0 (148.0–222.0)	73.2
Kidney	163.0 (129.0–193.0)	65.2
Liver	158.5 (129.0–194.0)	63.4
Ovary	35.5 (20.0–51.0)	14.2
Muscle	34.3 (30.0–41.0)	13.7
Pancreas	25.0 (25.0–40.0)	10.0

[a] Average values for four rabbits with range in parentheses. Average plasma concentration of TYR at time of sacrifice was 2.5 ng/ml (range 1.5–3 ng/ml).

ployed to determine the specificity of the TYR antibodies by competitive binding experiments. The only compounds that were partially recognized by the antibody were *p*-methoxyphenethylamine, *p*-acetoxyphenethylamine, dopamine, and 3-*O*-methyldopamine. Although dopamine and its metabolite 3-*O*-methyldopamine were weakly recognized (Table I), the endogenous levels of these two compounds in biological fluids are so low (25, 26) it is unlikely that they would interfere with the radioimmunoassay of TYR. An important aspect of the radioimmunoassay of TYR is that the major metabolites of TYR, (octopamine, *p*-hydroxyphenylacetic acid, *p*-hydroxyphenethanol, and *p*-hydroxymandelic acid) did not interfere (Table I).

The radioimmunoassay of TYR was used to analyze the plasma levels of TYR at different time intervals after the administration of TYR-^{14}C to rabbits. The disappearance of TYR from plasma followed a multiphasic pattern with a rapid decrease in the levels of TYR during the first 10 min. This pattern was similar to the disappearance of TYR from the heart previously described by Commarato *et al.* (27). However, the total ^{14}C (TYR equivalent) represented a much slower decline, accounting probably for the accumulation and excretion of the major metabolites of TYR such as *p*-hydroxyphenylacetic acid (Fig. 3). This phenomenon has been demonstrated with other phenethylamine derivatives such as methylphenidate (28) and the β-adrenergic blocking agent propranolol (29).

In preliminary studies, the radioimmunoassay was also used to measure the endogenous tissue levels of TYR in rabbits. Contrary to what has been previously reported (1), the highest concentration of TYR was found in the adrenals and spleen. The high concentration of TYR in the adrenals may explain the slow turnover of catecholamines and their precursors by the adrenal medulla (30–32).

Tyramine is inactivated mainly by monoamine oxidase. Under some pathological conditions such as hyperthyroidism (33), toxicosis of pregnancy (34), hyperkinesia (35), encephalitis (36), and certain liver malfunctions (37), a considerable decrease in monoamine oxidase activity had been observed. Thus, in such clinical situations, the determination of TYR levels in plasma and urine by radioimmunoassay may provide a valuable diagnostic tool.

Summary. Antibodies were prepared against tyramine. The antigen was prepared as follows: *p*-Aminohippuric acid was coupled to mBSA using a carbodiimide reagent. The amino group was diazotized and attached to the aromatic ring of TYR. The immunogen in Freund's complete adjuvant was injected into rabbits. The specificity of the resulting antibody was determined by radioimmunoassay. Using random-labeled TYR-^{3}H, TYR, its metabolites, phenethylamine analogs, catecholamines, and certain amino acids were evaluated by a competitive binding assay method. With this technique 4 ng of TYR inhibited the binding of TYR-^{3}H by 50%. The radioimmunoassay of TYR was used to measure the plasma, urine, and tissue levels of TYR in rabbits. The plasma disappearance curve of TYR revealed a biphasic pattern with $t_{1/2}$ of 2 min and 54 min. The highest concentration of TYR was found in adrenals and spleen. The factthat the major metabolites of TYR and a series of pharmacologically important sympathomimetics and catecholamines did not interfere, makes the radioimmunoassay of TYR a useful, simple, sensitive, and specific

method for the direct analysis of TYR in biological materials.

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