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Assay of tryptophan 2,3-dioxygenase using liver slices and high-performance liquid chromatography

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ABSTRACT

Liver tryptophan 2,3-dioxygenase (TDO) activity was determined by high-performance liquid chromatography. The enzyme activity was expressed as the sum of N-formyl-L-kynurenine (FK) and L-kynurenine (KYN) produced from L-tryptophan (TRY) by liver slices. FK and KYN were detected spectrophotometrically at 254 nm after their separation on a reversed-phase C_{18} column. KYN formation proceeded according to zero-order kinetics for at least 4 h with 15 m*M* TRY at 37°C. The apparent Michaelis constant was 1.2 m*M* TRY with a maximum velocity of 59 pmol min^{-1} mg^{-1} wet weight. The method was applied for TDO assay in mice treated with the organophosphorus acid triester diazinon. Kynurenine formamidase inhibition by diazinon resulted in reduced KYN formation, FK accumulation, and moderate TDO increase.

INTRODUCTION

Tryptophan 2,3-dioxygenase (EC 1.13.11.11) (TDO) catalyzes the conversion of L-tryptophan (TRY) into N-formyl-L-kynurenine (FK). The importance of this enzyme is underlined by its location at the beginning of the L-kynurenine (KYN) pathway of TRY metabolism. There is a need for a simple and reliable method for TDO assay since numerous pathological processes, nutritional status, or drugs alter the activity of this enzyme [1–3]. TDO assays are based on measurement of KYN formed by either enzyme- or acid-catalyzed hydrolysis of FK, the immediate TRY metabolite [4,5]. Formation of light-absorbing products of ascorbic acid oxidation or its products with TRY [6], dependence on an FK-hydrolyzing agent, and inability to distinguish between FK and KYN are the most serious shortcomings of the original spectrophotometric method [4]. Some of the problems can be partially overcome by using HPLC for KYN determination [7] or [^{14}C]TRY with a radiometric determination of [^{14}C]formic acid released from [^{14}C]FK [8]. All above-cited methods utilize tissue homogenates, fractionated to different extent by centrifugation, and consequently require additional components in order to maintain the *in vitro* TDO activity (*e.g.*, hemoglobin, ascorbic acid [4]).

This paper reports a new TDO assay using liver slices and simultaneous HPLC determination of FK and KYN formed from TRY. The method was examined for KYN, FK and TDO in mice treated with the organophosphorus acid triester diazinon.

EXPERIMENTAL

Chemicals

FK was obtained from Chemical Dynamics (South Plainfield, NJ, USA), L-[side-chain-2,3-^{3}H]TRY (55 Ci/mmol) from Amersham (Arlington Heights, IL, USA), silica plates (Fisherbrand Gel GF, 0.25- and 1-mm-thick lay-

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ers) and Acrodisc LC13 PVD filters from Fisher Scientific (Santa Clara, CA, USA). Diazinon [O,O-diethyl-O-(2-isopropyl-6-methyl-4-pyridiminyl) phosphorothioate] (>99%, confirmed by GC–MS) was a gift of Ciba-Geigy (Greensboro, NC, USA). All other chemicals were purchased from Sigma (St. Louis, MO, USA).

Animals

Swiss–Webster male mice (Simonsen, Gilroy, CA, USA) (22–35 g, eight to twelve weeks old) had free access to food (Purina Chow) and water at a 12-h dark–light cycle.

Liver slices

Mice were bled off by a cardiac puncture after cervical dislocation. The non-perfused livers were used throughout this study since perfusion of livers with isotonic NaCl did not have any effect on KYN/FK formation. Some livers were stored at −80°C for several months. The storage at −80°C for one month reduced the KYN/FK formation in slices by *ca.* 20%. Livers washed in isotonic sucrose were sectioned into cube-shaped slices by a scalpel. The weight and size of slices optimal for KYN formation were established for varying slice amounts (21–261 mg) of a uniform size (4–5 mg slices) and/or for one slice of a varying size (3–82 mg), respectively.

FK/KYN and TDO assay

Kinetics of KYN formation and the effects of O_2, slice preincubation, the manner of termination of the enzyme reaction, and TRY concentration on KYN formation were examined prior to establishing a protocol for the FK/KYN assay. Consequently, the assay was run under the following conditions: ten to fifteen pieces of liver slices (each of the average weight 4 ± 1 mg) were preincubated in 3 ml of Krebs solution for 30 min at 37°C. The preincubation medium was discarded and the enzyme reaction started by adding 1 ml of 15 m*M* TRY in Krebs solution. The reaction mixture was incubated at 37°C for 2 h. The reaction was stopped by immersing the reaction vials into an ice-bath and transferring the medium into a centrifuge tube containing 1 ml of precooled (−5°C) methanol. After 30 min standing on ice the mixture was centrifuged for 15 min at 5000 *g* and the protein precipitate discarded. The supernatant was filtered through a 0.2-μm Acrodisc filter prior to HPLC separation. TDO activity was defined as the sum of the amounts of FK and KYN formed per milligram of liver slice (wet weight) per min.

High-performance liquid chromatography

A Spectra-Physics Isochrom LC pump, a Rheodyne Model 7125 syringe loading sample injector (10-μl sample loop) and a Spectra Chrom 100 UV detector were the basic HPLC components (Spectra-Physics, San Jose, CA, USA). Samples were separated on a reversed-phase C_{18} column (30 mm × 4.6 mm I.D., 3-μm CR Pecosphere, Perkin-Elmer) with a mobile phase of 5% acetonitrile in 1 m*M* phosphate buffer, pH 2.4, at a flow-rate of 0.5 ml/min. Typically, 5 μl of the filtered reaction mixture (see above) were injected for analysis. KYN and FK detected at 254 nm were eluted at 5.3 ± 0.2 and 6.6 ± 0.2 min. They were quantified using a standard calibration curve.

FK/KYN confirmation

The identities of FK and KYN were confirmed by HPLC with FK and KYN as internal standards and using the phosphate buffer with varying acetonitrile concentrations (3–12%, v/v). The fractions from HPLC were collected and examined for FK/KYN by TLC (silica gel; *n*-propanol–*n*-butanol–NH_4OH–water, 4:2:0.01:4; KYN colored violet and FK yellow after a ninhydrin detection), enzymatically after kynurenine formamidase (KFase; EC 3.5.1.9) addition [4], and spectrophotometrically. FK/KYN was also confirmed radiometrically with [^{3}H]TRY used as a substrate for liver slices.

Application of the liver slices TDO HPLC assay

Mice were treated intraperitoneally with the KFase inhibitor diazinon [9] (1 and 240 mg/kg, applied in methoxytriglycol). The controls received 20 μl of the carrier solvent. The mice were

killed 36 h after diazinon administration. Slices prepared from livers pooled from two mice were incubated with TRY, and FK/KYN assayed as outlined before.

RESULTS AND DISCUSSION

The increase in KYN was linear up to 80 mg of the total weight of uniformly sized liver slices (4 ± 1 mg) (Fig. 1). The size of slices was the other important factor affecting KYN formation (Table I). The specific KYN production decreased with increasing size of slices, probably as a result of a slower substrate diffusion into the slices. Both parameters should be kept constant and within the weight (< 80 mg) and size (3–5 mg slices) range for good reproducibility of this method. The manual sectioning of livers into 1–1.5 mm side cubes with a scalpel accomplished these requirements.

Krebs solution was used for slice incubation, based on its reported satisfactory application in a study of quinolinic acid biosynthesis [10]. Saturation of the reaction mixture with O_2 had no effect on the rate of FK and KYN formation (Table I). Incubation of liver slices in Krebs solution at 37°C for 30 and 60 min prior to L-TRY addition enhanced slightly the FK/KYN formation (Table I). The resulting exudate did not change the rate of KYN formation when added back to the slices indicating that this increase in KYN was not caused by removal of an endogenous TDO inhibitor from the slices. More than 90% of KYN formed by slices was excreted into the media. Methanol precipitation of medium proteins prior to HPLC gave a higher KYN yield (*ca.* 1.40-fold) than the heat treatment (Table I).

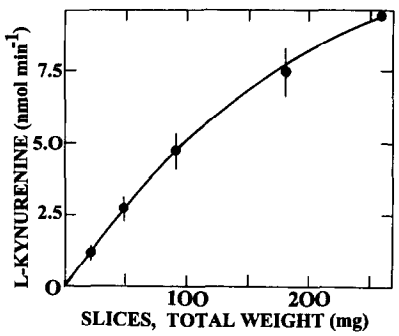

Fig. 1. L-Kynurenine formation by varying amounts of liver slices; 21–261 mg of the slices (each slice 4 ± 1 mg) were incubated with 15 m*M* TRY at 37°C for 2 h. Values are the means of two independent experiments with the range indicated by bars.

TABLE I

EFFECTS OF VARIOUS EXPERIMENTAL FACTORS ON KYN FORMATION BY MICE LIVER SLICES

Coefficient of variation of KYN formed was 8% (n = 6). Controls for O_2, ascorbic acid and hemoglobin were obtained with liver slices incubated under the standard conditions.

Factor	KYN formation	
	pmol min^{-1}	% of control
Slice weight		
3 mg	62	
5 mg	57	
10 mg	44	
19 mg	37	
82 mg	14	
O_2[a]		102
Preincubation time		
0 min		88
30 min		100
60 min		104
120 min		54
180 min		33
Termination of the reaction		
Thermal[b]		71
Methanol		100
Ascorbic acid (5 m*M*)		65
Hemoglobin (10 mg/l)		100

[a] Krebs solution saturated with O_2.
[b] 5 min at 100°C.

In contrast to liver homogenates or purified TDO preparations, liver slices did not require exogenous methemoglobin or ascorbic acid for TDO activation (Table I). KYN was formed immediately after TRY addition to the slices without the initial lag period which is characteristic for KYN formation in the former two preparations [4]. KYN was formed from TRY according to zero-order kinetics up to at least 4 h with 15 m*M* TRY at 37°C (Fig. 2). The apparent Michaelis constant (K_m) was 1.2 ± 0.1 mmol/l for TRY

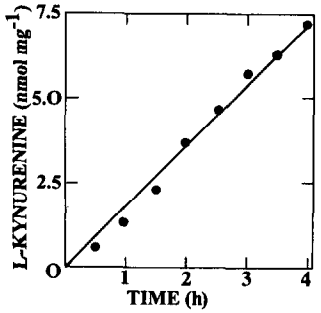

Fig. 2. Time course of L-kynurenine formation by liver slices. The slices (80 mg) were incubated with 15 m*M* TRY at 37°C. *k*, calculated by linear regression, was 28 ± 4 pmol mg^{-1} wet weight min^{-1} (r = 0.99).

with a maximum velocity (V_{max}) of 59 ± 0.7 pmol min^{-1} mg^{-1} wet weight (Fig. 3). K_m obtained with the slices is higher than the values reported for liver homogenates or purified liver preparations (0.1–0.4 mmol/l) [11]. It probably reflects the higher TRY requirements to support its transport into hepatocytes. Only a small amount of FK ($\leq$1 pmol mg^{-1} min^{-1}) was detected in the media, indicating a rapid FK hydrolysis by endogenous KFase with FK/KYN steady-state shifted toward KYN formation. TDO activity of slices prepared from fresh livers varied from 40 to 60 pmol min^{-1} mg^{-1} wet weight. The coefficient of variation of KYN formed in slices

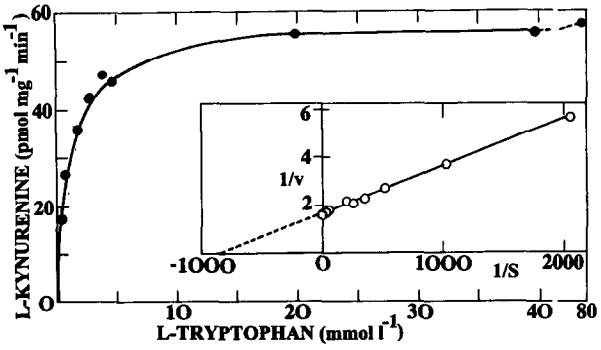

Fig. 3. Dependence of L-kynurenine formation in liver slices on L-tryptophan. Mice liver slices (80 mg) were incubated with varying concentrations of TRY (0.5–79 mmol/l) at 37°C for 2 h. Values were obtained from two independent experiments. Inset is Lineweaver–Burke plot: 1/*S* in mol^{-1}, 1/*v* in mg min $pmol^{-1}$. K_m and V_{max} were computed by the Enzfitter non-linear regression program for the IBM PC (Elsevier-Biosoft, Cambridge, UK).

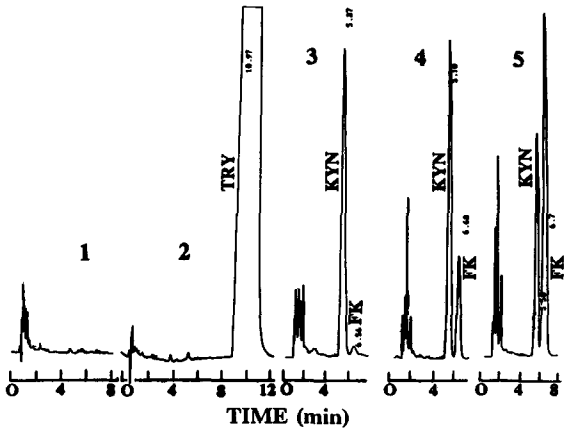

Fig. 4. Effects of diazinon treatment on L-kynurenine and N-formyl-L-kynurenine formation by mice liver slices. (1) Liver slices without TRY addition; (2) 15 m*M* TRY in Krebs solution without liver slices (5 μl); (3) control mice liver slices with 15 m*M* TRY; (4, 5) diazinon-treated mice (1 and 240 mg/kg) liver slices with 15 m*M* TRY.

prepared from the same liver was 8% (n = 6). The lowest measurable TDO activity based on KYN and FK detection limits (signal-to-noise ratio of 3) would be 1 pmol min^{-1} mg^{-1} wet weight.

The new method was examined for TDO assay in mice treated with the organophosphorus insecticide diazinon. This experiment demonstrated the major advantage of this method, *i.e.* simultaneous determination of FK and KYN. KFase inhibition by diazinon resulted in FK accumulation (Fig. 4) while KYN formation was reduced. TDO activity was moderately enhanced, probably due to corticosteroid induction by diazinon [12,13].

A greater structural integrity of liver slices compared to homogenates and purified enzyme preparations and discrimination between FK and KYN contributions in defining TDO activity are the major advantages of this method. Its simplicity, sensitivity, and accuracy makes it suitable for investigating effects of drugs, pathological conditions, environmental toxicants, or nutritional factors on the entry reactions of the KYN pathway of TRY metabolism.

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