Biochem. J. (1980) **189**, 393–405
Printed in Great Britain

Stereospecificity of hepatic L-tryptophan 2,3-dioxygenase

Yasuyoshi WATANABE, Motokazu FUJIWARA,* Ryotaro YOSHIDA and Osamu HAYAISHI
Department of Medical Chemistry, Kyoto University Faculty of Medicine, Kyoto 606, Japan

(*Received 17 December 1979*)

Tryptophan 2,3-dioxygenase [L-tryptophan–oxygen 2,3-oxidoreductase (decyclizing), EC 1.13.11.11] has been reported to act solely on the L-isomer of tryptophan. However, by using a sensitive assay method with D- and L-[*ring*-2-^{14}C]tryptophan and improved assay conditions, we were able to demonstrate that both the D- and L-stereoisomers of tryptophan were cleaved by the supernatant fraction (30000**g**, 30min) of liver homogenates of several species of mammals, including rat, mouse, rabbit and human. The ratio of activities toward D- and L-tryptophan was species variable, the highest (0.67) in ox liver and the lowest (0.07) in rat liver, the latter being hitherto exclusively used for the study of hepatic tryptophan 2,3-dioxygenase. In the supernatant fraction from mouse liver, the ratio was 0.23 but the specific activity with D-tryptophan was by far the highest of all the species tested. To identify the D-tryptophan cleaving enzyme activity, the enzyme was purified from mouse liver to apparent homogeneity. The specific activities toward D- and L-tryptophan showed a parallel rise with each purification step. The electrophoretically homogeneous protein had specific activities of 0.55 and 2.13μmol/min per mg of protein at 25°C toward D- and L-tryptophan, respectively. Additional evidence from heat treatment, inhibition and kinetic studies indicated that the same active site of a single enzyme was responsible for both activities. The molecular weight (150000), subunit structure ($\alpha_2\beta_2$) and haem content (1.95mol/mol) of the purified enzyme from mouse liver were similar to those of rat liver tryptophan 2,3-dioxygenase. The assay conditions employed in the previous studies on the stereospecificity of hepatic tryptophan 2,3-dioxygenase were apparently inadequate for determination of the D-tryptophan cleaving activity. Under the assay conditions in the present study, the purified enzyme from rat liver also acted on D-tryptophan, whereas the pseudomonad enzyme was strictly specific for the L-isomer.

Tryptophan 2,3-dioxygenase [L-tryptophan–oxygen 2,3-oxidoreductase (decyclizing), EC 1.13.11.11] is a haem-containing enzyme that catalyses the oxygenative ring cleavage of L-tryptophan. This enzyme was isolated, purified and characterized from rat liver (Knox & Mehler, 1950; Schimke, 1970; Schutz & Feigelson, 1972) and also from *Pseudomonas* (Hayaishi & Stanier, 1951; Poillon *et al.*, 1969; Ishimura, 1970). It has been reported to be highly specific for the L-isomer of tryptophan (Knox & Mehler, 1950; Tanaka & Knox, 1959; Civen & Knox, 1960; Schimke, 1970; Ishimura, 1970; Feigelson & Brady, 1974), so that the term 'L-tryptophan–oxygen 2,3-oxidoreductase' has exclusively been employed up to the present (*Enzyme Nomenclature*, 1979).

Mammalian tryptophan 2,3-dioxygenase has so far been found only in the liver (Knox, 1955*a*), whereas indoleamine 2,3-dioxygenase, another haem-containing dioxygenase that cleaves the indole ring of tryptophan (Higuchi & Hayaishi, 1967; Yamamoto & Hayaishi, 1967), is widely distributed in various tissues of mammals except the liver (Hayaishi *et al.*, 1975). Indoleamine 2,3-dioxygenase acts on both D- and L-tryptophan and other indoleamines (Hirata & Hayaishi, 1972; Shimizu *et al.*, 1978). These two dioxygenases differ from each other with regard to molecular properties (Schutz & Feigelson, 1972; Shimizu *et al.*, 1978) and the reaction mechanism (Brady *et al.*, 1971; Hirata & Hayaishi, 1971, 1975; Hirata *et al.*, 1977; Ohnishi *et al.*, 1977; Taniguchi *et al.*, 1979).

Abbreviations used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid; SDS, sodium dodecyl sulphate.

* Present address: Department of Pharmacology, Kyoto University Faculty of Medicine, Kyoto 606, Japan.

0306-3275/80/090393-13$01.50/1 © 1980 The Biochemical Society

In the present study, an improved, sensitive and stereospecific assay method for the D-tryptophan cleaving activity was developed. With this assay, D-tryptophan cleaving activity was found in the supernatant fraction of liver homogenates of several species of mammals. This finding prompted us to investigate whether or not indoleamine 2,3-dioxygenase exists in the liver, or whether, in contrast to the previous reports, tryptophan 2,3-dioxygenase acts on both stereoisomers of tryptophan. The enzyme was purified from mouse liver, and was shown to cleave both stereoisomers of tryptophan. The molecular and catalytic properties of the purified enzyme were similar to those of rat liver tryptophan 2,3-dioxygenase, which was also shown to act on both stereoisomers under our assay conditions. The results presented in this paper indicate that hepatic tryptophan 2,3-dioxygenase from mammalian sources metabolizes both D- and L-isomers of tryptophan. The reason for the discrepancy between our results and the results of previous studies is also discussed.

Experimental

Chemicals

Commercial sources of reagents were as follows: DL-[*ring*-2-^{14}C]tryptophan (35 Ci/mol) and DL-[*benzene*-U-^{14}C]tryptophan (45 Ci/mol) from Schwartz/Mann and The Radiochemical Centre, Amersham, respectively; L-tryptophan (A grade) and D-tryptophan, from Ajinomoto Co.; 5-hydroxy-L- and 5-hydroxy-D-tryptophan, tryptamine hydrochloride, serotonin creatinine sulphate, indole-3-acetic acid, indole-3-acrylic acid, and D- and L-kynurenine, from Sigma; haematin, from Calbiochem; Hepes, from Dohjin Chemicals Co.; standard solutions of copper and iron, from Wako Pure Chemical Industries; polyamide, from Woelm; precoated cellulose glass plates and cellulose powder (Avicel), from Merck; hog kidney D-amino acid oxidase, beef liver catalase and a standard protein kit for molecular weight determination (Combithek), from Boehringer Mannheim. All other chemicals were obtained commercially and were used without further purification unless specified.

Preparation of substrates

D-Tryptophan was recrystallized twice from hot methanol. The amount of contamination by L-tryptophan in the recrystallized sample was estimated to be 0.095% according to the method described previously (Snell *et al.*, 1937; Prescott *et al.*, 1949). DL-[^{14}C]Tryptophan was purified by Dowex 50W (X2; H^+ form) column chromatography as described previously (Ohnishi *et al.*, 1977) and was then resolved into radioactive D- and L-tryptophan by cellulose column chromatography with a solvent system of butan-1-ol/pyridine/water (4:4:1, by vol.) (M. Shibata, M. Fujiwara & O. Hayaishi, unpublished work).

Animals

Livers were obtained from the following species: goat, dog, rat (Sprague–Dawley), mouse (Slc:ICR) and rabbit, from the Institute of Laboratory Animals, Kyoto University; ox and pig, from the slaughterhouse (Kyoto). Monkey (*Macaca fuscata fuscata*) livers were provided by Dr. K. Takahashi of the Primate Research Institute, Kyoto University. Human liver (0.3 g obtained at autopsy from a patient who died of cerebrovascular haemorrhage) was provided by Dr. Y. Hamashima of the Department of Pathology, Kyoto University Faculty of Medicine. Animals were adult and male, except for the rats, which were all female.

Enzymes

Formamidase was prepared from rat liver by the method of Knox (1955*b*) and was further purified as described previously (Hirata *et al.*, 1977). The final preparation of the enzyme had a specific activity of 24 μmol/min per mg of protein at 25°C with *N*-formyl-L-kynurenine as substrate. This enzyme also acts on *N*-formyl-D-kynurenine (Loh & Berg, 1973). The purified tryptophan 2,3-dioxygenases from rat liver and *Pseudomonas fluorescens* were provided by Dr. Y. Ishimura and Dr. R. Makino of the Department of Biochemistry, School of Medicine, Keio University. Specific activities of these enzymes were 1.1 and 3.4 μmol/min per mg of protein at 25°C under the standard assay conditions (Schutz & Feigelson, 1972; Ishimura, 1970), respectively. Superoxide dismutase was purified from bovine erythrocytes by the method of McCord & Fridovich (1969). Specific activity was 3300 units/mg of protein at 25°C.

Buffers

Potassium phosphate buffers at pH 6.5 containing various concentrations of KCl and L-tryptophan were used during the purification of tryptophan 2,3-dioxygenase from mouse liver. The concentrations of the three compounds were varied as follows: buffer A, 10 mM-potassium phosphate/0.14 M-KCl/10 mM-L-tryptophan; buffer B, 10 mM/0.90 M/10 mM; buffer C, 0.10 M/0.50 M/10 mM; buffer D, 0.70 M/0.50 M/10 mM; buffer E, 10 mM/0/10 mM; buffer F, 0.10 M/0.50 M/1.0 mM. All buffers were saturated with N_2 to stabilize the enzyme as described by Schimke (1970) for rat liver tryptophan 2,3-dioxygenase. Buffers used after purification step 2 were bubbled with N_2 followed by 100% CO, as the addition of CO was found to be most effective in stabilizing mouse liver enzyme.

Pretreatment of enzyme before assay

To measure the D- and L-tryptophan cleaving activities of the enzyme at each purification step, L-tryptophan and CO added as stabilizers were removed by the following method. The enzyme preparation (1 ml) was applied to a column (1.0 cm × 25 cm) of Sephadex G-25 equilibrated with 0.5 M-KCl in 10 mM-potassium phosphate buffer (pH 7.0) and was eluted with the same buffer at a flow rate of 0.3 ml/min. Fractions (1 ml) were collected. The enzyme, free from tryptophan and CO, was eluted in tubes 9–12 and tryptophan was eluted thereafter (tubes 18 onwards). After purification step 3, the eluted enzyme exhibited an absorption spectrum with a Soret peak at 406 nm and minor peaks at around 500 and 630 nm. These peaks were not altered either by the addition of 1 mM-ammonium persulphate or by gentle bubbling of CO, indicating that the haem of the enzyme was in the ferric form.

Assay of enzyme activity

D- and L-Tryptophan cleaving activities were measured by the following two procedures. All assays were done at 25°C except those described in Table 1, which were done at 37°C. Procedure B is about 100-fold more sensitive than procedure A. Procedure A was used to monitor the time course of the reaction, whereas procedure B was used when the crude enzyme was assayed and when high sensitivity was required for the assay of the purified enzyme. The results with the purified enzyme were essentially identical with both procedures.

Procedure A. The formation of *N*-formylkynurenine was followed spectrophotometrically at 321 nm ($\varepsilon = 3.75\,mM^{-1}\cdot cm^{-1}$) with a Shimadzu recording spectrophotometer model UV-300. The standard reaction mixture (1.0 ml) contained 50 mM-Hepes/NaOH (pH 7.3), 5.0 mM-D-tryptophan or 2.0 mM-L-tryptophan, 10 μM-Methylene Blue, 5.0 mM-ascorbic acid, 1.0 μM-catalase, and the enzyme. Substrate or enzyme was omitted in the blank cuvette.

Procedure B. The rate of [^{14}C]formate release from [*ring*-2-^{14}C]tryptophan was measured by the method originally reported by Peterkofsky (1968) and modified by Taniguchi *et al.* (1977). The standard reaction mixture (0.2 ml) contained 50 mM-Hepes/NaOH (pH 7.3), 25 mM-sodium formate, 5.0 mM-D-[*ring*-2-^{14}C]tryptophan (500 c.p.m./nmol) or 2.0 mM-L-[*ring*-2-^{14}C]tryptophan (300 c.p.m./nmol), 3.3 μg of formamidase, 10 μM-Methylene Blue, 5.0 mM-ascorbic acid, 1.0 μM-catalase and the enzyme.

The photodissociation of the CO–enzyme–L-tryptophan complex was performed by the method described by Gibson (1959) and modified by Taniguchi *et al.* (1979). To obtain the CO–enzyme–L-tryptophan complex free from dithionite, column chromatography using Sephadex G-25 was carried out in the dark as described above, except that the buffer contained 10 mM-L-tryptophan and was saturated with CO gas.

One unit of enzyme activity was defined as the amount producing 1 μmol of *N*-formylkynurenine/min at 25°C. Specific activity was expressed as units/mg of protein.

Purification of tryptophan 2,3-dioxygenase from mouse liver

All subsequent procedures were performed at 0–4°C and centrifugations were carried out at 10000 ***g*** for 20 min unless stated otherwise.

Step 1: crude extracts. One thousand male ICR mice, each weighing approx. 36 g, were injected intraperitoneally twice with a mixture, in 0.9% NaCl (4 ml), of 36 mg of L-tryptophan and 1.35 mg of hydrocortisone acetate/mouse with a 4 h interval. The animals were killed 8 h after the first inducing dose and the livers were removed and kept frozen at −70°C. The frozen livers (1400 g) were thawed and homogenized in 3 vol. of buffer A by using a Polytron homogenizer. Homogenates were centrifuged at 13000 ***g*** for 20 min and the supernatant obtained was centrifuged at 100000 ***g*** for 1 h. The resulting supernatant (3600 ml) was referred to as crude extracts.

Step 2: $(NH_4)_2SO_4$ fractionation. The supernatant was fractionated by the addition of solid $(NH_4)_2SO_4$. The precipitate formed between 33 and 47% saturation were dissolved in buffer C (450 ml) and dialysed for 3 h against two changes of 10 litres of buffer F.

Step 3: hydroxyapatite column chromatography. After insoluble material had been removed by centrifugation, the clear supernatant was diluted approx. 2-fold with buffer C to give a final protein concentration of 53 mg/ml. Small amounts (25 mg) of sodium dithionite were added to the enzyme solution (1002 ml) and then 100% CO was bubbled gently for 10 min. Saturation of CO in the solution was checked spectrophotometrically as judged by the increase in A_{419} owing to the formation of the CO-bound form of the contaminating haemoglobin. To obtain a more adequate flow rate, the enzyme solution was divided into four parts (250 ml each). Each part was then applied to a column (4 cm × 3 cm) of hydroxyapatite equilibrated with buffer C; the enzyme was eluted with buffer D. The active enzyme fractions (60 ml) were combined and diluted 5-fold with buffer E.

Step 4: second hydroxyapatite column chromatography. The enzyme solution was applied to a column (4 cm × 8 cm) of hydroxyapatite equilibrated with buffer C. The column was washed with 500 ml of buffer C. A linear gradient elution was performed with 300 ml each of buffers C and D. The

active enzyme fractions were eluted at around 0.35 M-potassium phosphate buffer. To concentrate the enzyme, the following procedures were carried out: the enzyme solution was diluted with 4 vol. of buffer E, applied to a column (3 cm × 2 cm) of hydroxyapatite equilibrated with buffer C and the enzyme was eluted with buffer D.

Step 5: gel filtration on Bio-Gel A-0.5 m. The concentrated enzyme fraction (7.5 ml, 236 mg of protein) was applied to a column (2.5 cm × 100 cm) of Bio-Gel A-0.5 m equilibrated with buffer A. Elution was performed with the same buffer. Those enzyme fractions having a specific activity greater than 0.22 unit/mg of protein were combined.

Step 6: DEAE-cellulose column chromatography. The combined enzyme solution (71 ml) was directly applied to a column (3.2 cm × 7 cm) of DEAE-cellulose equilibrated with buffer A. The column was washed with 100 ml of buffer A and then linear gradient elution was performed with 150 ml each of buffers A and B. The active enzyme fractions were eluted at about 0.4 M-KCl.

Step 7: third hydroxyapatite column chromatography. The combined enzyme solution (36 ml) was directly applied to a column (3 cm × 3 cm) of hydroxyapatite equilibrated with buffer C. The column was washed with the same buffer. A linear gradient elution was performed with 75 ml each of buffers C and D. The active enzyme fractions were concentrated by using a column (1.2 cm × 2 cm) of hydroxyapatite as described in step 4.

Step 8: Sephadex G-200 gel filtration. The concentrated sample (3 ml, 6.3 mg of protein) was applied to a column (2.2 cm × 60 cm) of Sephadex G-200 equilibrated with buffer A. Elution was performed with the same buffer at a flow rate of about 18 ml/h. The active enzyme fractions having a specific activity greater than 1.6 units/mg of protein were combined.

Step 9: second DEAE-cellulose column chromatography. The enzyme solution (32 ml) was applied to a column (2 cm × 4 cm) of DEAE-cellulose equilibrated with buffer A. After the column had been washed with buffer A (100 ml), a linear gradient elution was performed with 100 ml each of buffers A and B. Then the active enzyme fractions were combined, concentrated as described in step 4 and stored at −70 °C.

Polyacrylamide-gel electrophoresis

Polyacrylamide-gel electrophoresis [7.5% (w/v) gel] was performed at pH 8.3 by the method of Davis (1964). Electrophoresis on polyacrylamide [10% (w/v)] gels containing 0.1% SDS was performed by the method of Weber & Osborn (1969). RNA polymerase (mol.wt. of subunits α, β, and β': 39000, 155000 and 165000, respectively), bovine serum albumin (mol.wt. 67000), soya bean trypsin inhibitor (mol.wt. 21500), and indoleamine 2,3-dioxygenase purified from rabbit small intestine (mol.wt. 40000) (Shimizu *et al.*, 1978) were used as marker proteins for the determination of the molecular weight. Gels were stained with 0.25% Coomassie Blue R-250 and destained by diffusion in 7% (v/v) acetic acid/10% (v/v) methanol.

Determination of molecular weight

Analytical gel filtration was carried out at 4 °C on a column (2.0 cm × 70 cm) of Sephadex G-200 previously equilibrated with buffer A. The molecular weight of the enzyme was determined by the gel filtration method of Andrews (1965). The following proteins were used as standards; beef liver catalase (mol.wt. 250000), yeast alcohol dehydrogenase (mol.wt. 150000), hog kidney D-amino acid oxidase (mol.wt. 100000), bovine serum albumin (mol.wt. 67000), and bovine pancreatic α-chymotrypsinogen (mol.wt. 24500).

Other determinations

Protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Since tryptophan interferes with the protein determination, proteins were precipitated by the addition of 10% (w/v) trichloroacetic acid before the measurement.

The haem content of the purified enzyme was determined as the pyridine–ferrohaemochrome according to the method of Paul *et al.* (1953) using an absorbance coefficient of 34.4 $mM^{-1} \cdot cm^{-1}$ at 557 nm.

The iron and copper contents of the purified enzyme dialysed for 3 h against 3 litres of buffer A were measured by using a Varian Techtron model 1100 atomic absorption spectrophotometer with a carbon rod atomizer model 63. $FeCl_3$ and haemoglobin were employed as standards for iron determinations and $CuCl_2$ and tyrosinase for copper.

Radioactivity was determined with Packard liquid-scintillation counter model 3385 as described previously (Ohnishi *et al.*, 1977).

Results

Effects of various assay conditions on D- and L-tryptophan cleaving activities

Various activators were examined for the optimum assay conditions of the ferrihaem form of tryptophan 2,3-dioxygenase purified from mouse liver, since it has been demonstrated that pseudomonad and rat liver tryptophan 2,3-dioxygenases were inactive in the ferrihaem state and required reductive activation to be converted to the active ferrohaem state for the maximum enzyme activity (Tanaka & Knox, 1959; Civen & Knox, 1960; Knox & Ogata, 1965; Schimke *et al.*, 1965*a*;

Schimke, 1970; Ishimura, 1970; Brady *et al.*, 1971).

Previous investigators, who reported that rat liver tryptophan 2,3-dioxygenase did not act on D-tryptophan, used a H_2O_2-generating system (Knox & Mehler, 1950; Civen & Knox, 1960) or ascorbate (Tanaka & Knox, 1959; Schimke, 1970) as a sole activator. The effects of various concentrations of such activators on D- and L-tryptophan cleaving activities of the enzyme purified from mouse liver are presented in Figs. 1 and 2. As shown in Figs. 1*a* and 1*b*, with either D- or L-tryptophan as substrate, the enzyme reaction proceeded linearly in the absence of activators after a lag period of 5–10min (Fig. 1, curves 1) as reported for L-tryptophan cleavage by the rat liver and pseudomonad enzymes (Tanaka & Knox, 1959). Dose-dependent stimulation of the reaction rate and shortening of the lag time by ascorbate were also observed (Fig. 1, curves 2–4). The concentration of ascorbate required for half-maximal activation was about 25μM in both cases. At greater concentrations of ascorbate, the results were complicated. When L-tryptophan was used as substrate, above 0.1 mM-ascorbate no further stimulation was observed (Fig. 1*a*, curves 5–7) and above 1.0 mM-ascorbate inactivated the enzyme (results not shown). However, above 0.25 mM-ascorbate was inhibitory with D-tryptophan as substrate (Fig. 1*b*, curves 5–7). The addition of appropriate amount (2.0nM) of catalase in the presence of 0.5mM-ascorbate restored the D-tryptophan cleaving activity (Fig. 1*b*, dotted curve 8) whereas the L-tryptophan cleaving activity was rather inhibited under the same conditions (Fig. 1*a*, dotted curve 8), indicating that the D-tryptophan cleaving activity is more easily inactivated by H_2O_2 (probably generated by the auto-oxidation of ascorbate) compared with the activity toward the L-isomer. Marked inhibition specific for the D-tryptophan cleaving

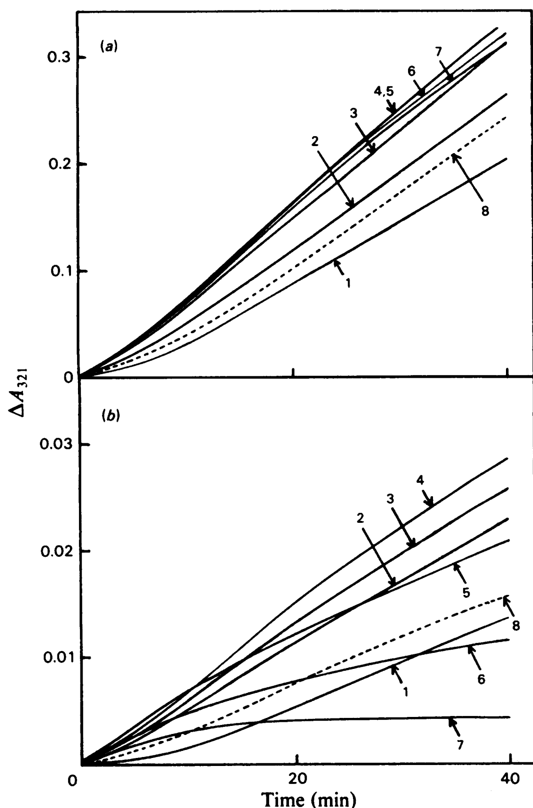

Fig. 1. *Effect of ascorbate concentration on the time course of the enzyme reaction with L-tryptophan (a) or D-tryptophan (b) as substrate*

The purified enzyme from mouse liver (1.82μg) was assayed at 25°C by using procedure A with ascorbate alone as an activator. Ascorbic acid concentrations: 1, 0mM; 2, 0.025mM; 3, 0.050mM; 4, 0.10mM; 5, 0.25mM; 6, 0.50mM; 7, 1.0mM; 8, 0.50mM + 2.0nM-catalase.

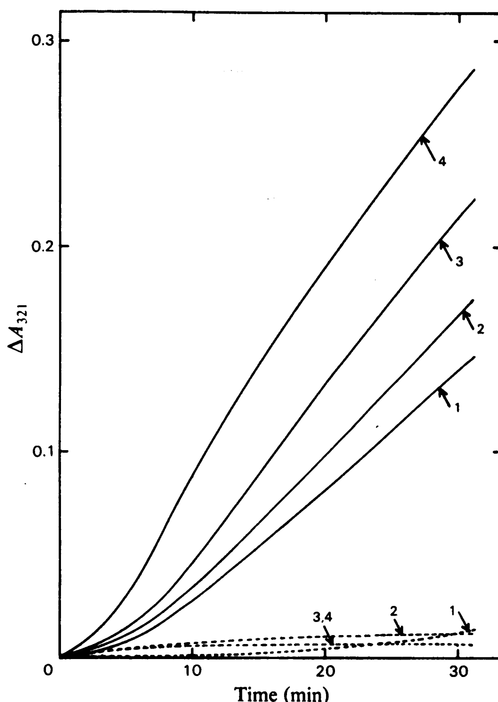

Fig. 2. *Time course of the enzyme reaction with an H_2O_2-generating system as a sole activator*

The purified enzyme from mouse liver (1.82μg) was assayed at 25°C by using procedure A with 0.2mM-DL-alanine and various concentrations of pig kidney D-amino acid oxidase: 1, 0mU; 2, 0.1mU; 3, 0.5mU; 4, 2.0mU (1U = 1μmol of H_2O_2 generated/min at 25°C). ——, L-tryptophan as substrate; ----, D-tryptophan as substrate.

activity was observed by using a H_2O_2-generating system (Fig. 2) or H_2O_2 itself (0.1–1.0μM, results not included). When H_2O_2 was generated enzymatically under conditions similar to those described by Knox & Mehler (1950), the D-tryptophan cleaving activity was hardly detectable (Fig. 2, dotted curve 3), whereas the L-tryptophan cleaving activity was somewhat stimulated (Fig. 2, curve 3). These results suggest that the D-tryptophan cleaving activity was not sufficiently activated by such activators. Therefore, a Methylene Blue/ascorbate/catalase system, as used for the assay of indoleamine 2,3-dioxygenase (Yamamoto & Hayaishi, 1967), was tested for the assay of both activities because this system was expected to activate the enzyme without the involvement of H_2O_2.

Fig. 3 shows a comparison of the effect of the activators on the time course of the reaction catalysed by the enzyme purified from mouse liver. When L-tryptophan was used as substrate (Fig. 3*a*), at the optimum concentrations of Methylene Blue (10μM), ascorbate (5.0mM) and catalase (1.0μM) (curve 3), the lag time was abolished and the reaction rate increased to about 3-fold that obtained without the activators (Fig. 3*a*, curve 1) and to approx. 1.5-fold that obtained at the optimum concentration of ascorbate alone (Fig. 3*a*, curve 2). The reaction rate with these three activators was nearly equal to that obtained by the photodissociation of the CO–enzyme–L-tryptophan complex (Fig. 3*a*, dotted curve 4). Since the reaction rate obtained by photodissociation was considered to be that obtained from the fully active ferrohaem form of the enzyme (Taniguchi *et al.*, 1979), the rate with the three activators indicated nearly full activity of the enzyme. Therefore, the assay conditions with the three activators are better than the previous assay conditions with ascorbate alone, even for the L-tryptophan cleaving activity. On the other hand, when D-tryptophan was used as substrate (Fig. 3*b*), the effect of these three activators was much more pronounced. In the presence of these activators (Fig. 3*b*, curve 3), the lag time was abolished and the reaction rate was much higher than that obtained with (Fig. 3*b*, curve 2) or without (Fig. 3*b*, curve 1) ascorbate. In the presence of such a large amount (1μM) of catalase, both Methylene Blue and ascorbate were essential for cleavage of both D- and L-tryptophan; activity was much diminished if either was omitted. When catalase was omitted, the initial rate of the reaction was scarcely altered but the reaction rate decreased with time. The concentrations of the three activators required for half-maximal activities toward D- and L-tryptophan were

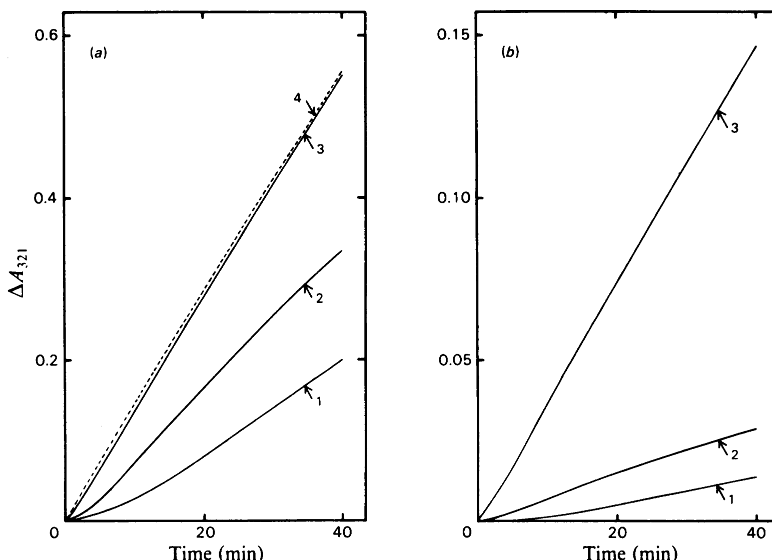

Fig. 3. *Time course of the enzyme reaction under various conditions with 2.0mM-L-tryptophan (a) or 5.0mM-D-tryptophan (b) as substrate*

The purified enzyme from mouse liver (1.82μg) was assayed at 25°C by using procedure A in a reaction mixture (1.0ml) containing 50mM-Hepes/NaOH (pH7.3), substrate and various activators: 1, no addition; 2, 0.1mM-ascorbate; 3, 10μM-Methylene Blue, 5.0mM-ascorbate and 1.0μM-catalase. Curve 4 in (*a*) indicates the time course of the reaction under the same conditions as curve 1 except that the reaction was initiated by the photodissociation of the CO–enzyme–L-tryptophan complex as described in the text.

as follows: Methylene Blue, 0.8 and 0.6 μM; ascorbate, 0.4 and 0.35 mM; catalase, 50 and 38 nM, respectively.

Haematin was reported to be an effective activator of the conversion of the apoenzyme into the holoenzyme for rat liver tryptophan 2,3-dioxygenase (Feigelson & Greengard, 1961; Knox & Ogata, 1965). However, for the mouse liver enzyme, the extent of activation by the addition of haematin (0.1–1.0 μM) to the incubation mixture containing ascorbate alone (0.1 mM) or the three activators was within 10% of the D- and L-tryptophan cleaving activities in the supernatant of the liver and also within 10% of both activities in liver induced by the administration of L-tryptophan and hydrocortisone, indicating that the apoenzyme was at most 10% of the enzyme in liver extract and in the starting material of the enzyme purification. Further, a requirement of haematin for either activity was not observed with the purified enzyme or the enzyme at any stage of purification under the standard assay conditions. Haematin was required for both activities only when the enzyme activity was determined in an incubation mixture containing a greater concentration (>0.5 mM) of ascorbate as a sole activator and when the enzyme was purified without CO, especially at the hydroxyapatite column step.

D- and L-Tryptophan cleaving activities in mammalian liver

The apparent D- and L-tryptophan cleaving activities of the supernatant fraction (30000 *g*, 30 min) of liver homogenates were measured by using procedure B with D- and L-[*ring*-2-^{14}C]tryptophan as substrates. Significant activities were observed in the supernatant of all mammalian livers examined. The ratio of D- and L-tryptophan cleaving activities was variable (0.07–0.67) depending on the species of animals used (Table 1). The lowest ratio was detected in the supernatant of rat liver, which has been exclusively used as the source of hepatic tryptophan 2,3-dioxygenase by previous investigators (Knox & Mehler, 1950; Tanaka & Knox, 1959; Civen & Knox, 1960; Schimke, 1970; Schutz & Feigelson, 1972; Feigelson & Brady, 1974). The ratio of the two activities in the supernatant of mouse liver was 0.23, and the specific activities were the highest among the mammalian livers tested. Therefore, mouse liver was used for further investigations to determine the origin of the D-tryptophan cleaving activity.

Induction of both activities

Administration of hydrocortisone and/or L-tryptophan was reported to induce tryptophan 2,3-dioxygenase in rat liver (Knox & Mehler, 1950; Schimke *et al.*, 1965*b*). Hydrocortisone acetate alone (37.5 μg/g body wt.), L-tryptophan alone (1 mg/g body wt.) or both were given to mice (five in each group) intraperitoneally, and 4 h later the specific enzyme activity for L-tryptophan of the supernatant of the liver was elevated 3-fold (342 pmol/min per mg of protein at 25°C), 2.5-fold (265 pmol/min per mg) and 6-fold (640 pmol/min per mg), respectively, as compared with that (108 pmol/min per mg) of the control animals injected with saline. The specific activity for the

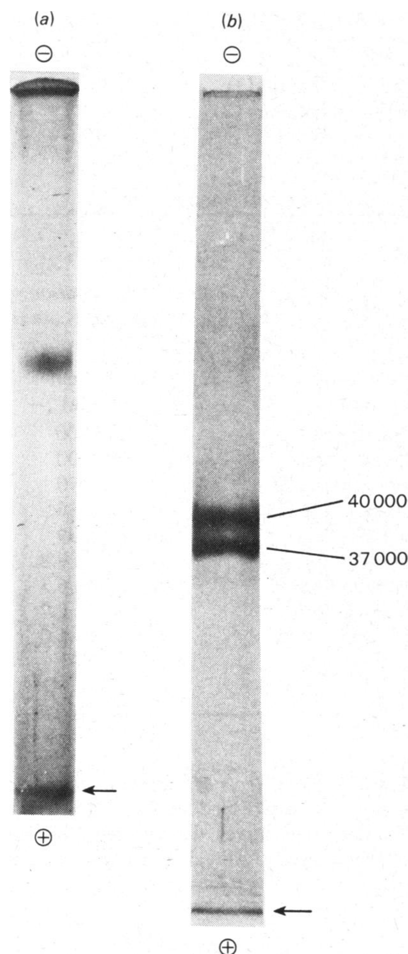

Fig. 4. *Polyacrylamide-gel electrophoreses of the purified enzyme*
(*a*) The purified enzyme (10 μg) was applied to a 7.5% (w/v) polyacrylamide gel at pH 8.3, and electrophoresed toward the anode at the bottom. (*b*) The molecular weights of the subunits were estimated by electrophoresis in a 10% (w/v) polyacrylamide gel containing 0.1% SDS. The enzyme (15 μg) was treated with SDS before electrophoresis. After electrophoreses, the gels were stained with Coomassie Blue. The arrows indicate the position of the tracking dye.

Table 1. *D- and L-tryptophan cleaving activities of the supernatant fractions of liver homogenates from various mammals*
Livers were homogenized with 2 vol. of buffer A without L-tryptophan. The homogenates were centrifuged at 30000 *g* for 30 min. An aliquot (100 μl, 4–5 mg of protein) of the supernatant was assayed at 37°C for 30 min by using procedure B as described in the Experimental section. The values represent means ± S.D. The number of experimental animals is shown in parentheses.

Species	Cleaving activity (pmol/min per mg of protein) with: D-Trp	L-Trp	D-Trp cleaving activity (% of L-Trp cleaving activity)
Ox (3)	10.6 ± 3.1	16.1 ± 5.1	66.5 ± 9.9
Goat (1)	10.0	36.0	27.8
Human (1)	9.2	36.0	25.4
Pig (3)	12.3 ± 1.4	50.7 ± 13.7	25.0 ± 3.2
Mouse (3)	56.6 ± 11.6	244 ± 45.8	23.3 ± 3.3
Dog (3)	12.1 ± 7.7	64.4 ± 40.2	18.6 ± 0.7
Rabbit (3)	18.5 ± 2.9	146 ± 16.5	12.7 ± 1.5
Monkey (3)	1.9 ± 0.5	24.7 ± 7.9	7.6 ± 0.6
Rat (3)	10.9 ± 3.2	159 ± 17.0	6.7 ± 1.3

Table 2. *Purification of tryptophan 2,3-dioxygenase from mouse liver*
All values are the averages of duplicate determinations.

Step	Protein (mg)	Specific activity for L-tryptophan (units/g)	Yield (%)	D-Trp cleaving activity (% of L-Trp cleaving activity)
1. Crude extracts	150000	0.713	100	24
2. $(NH_4)_2SO_4$ ppt.	53100	2.07	103	26
3. Hydroxyapatite I	1590	45.2	67	25
4. Hydroxyapatite II	236	181	40	28
5. Bio-Gel A-0.5 m	109	275	28	—
6. DEAE-cellulose I	29.8	611	17	29
7. Hydroxyapatite III	6.3	1460	8.6	25
8. Sephadex G-200	2.7	1690	4.2	—
9. DEAE-cellulose II	1.0	2130	2.0	26

Table 3. *The effects of various compounds on D- and L-tryptophan cleaving activities of the purified enzyme*
The assay was performed at 25°C for 10 min by using procedure B as described in the Experimental section. When indole derivatives were tested, the concentrations of substrates D- and L-tryptophan were 0.96 mM and 0.21 mM respectively. All values are the mean of two separate incubations that differed by less than 5%.

Compound	Concentration (mM)	Inhibition (%) of cleavage of: D-Trp	L-Trp
N-Ethylmaleimide	1.0	58	45
Iodoacetic acid	1.0	12	7
KCN	0.01	39	48
NaN_3	10	33	32
Superoxide dismutase	0.006	0	0
	0.012	0	0
Tiron	1.0	0	0
	10	2	6
5-Hydroxy-L-tryptophan	0.1	40	53
5-Hydroxy-D-tryptophan	0.1	26	29
Tryptamine	0.1	16	11
Serotonin	1.0	36	37
Indole-3-acetic acid	1.0	19	27

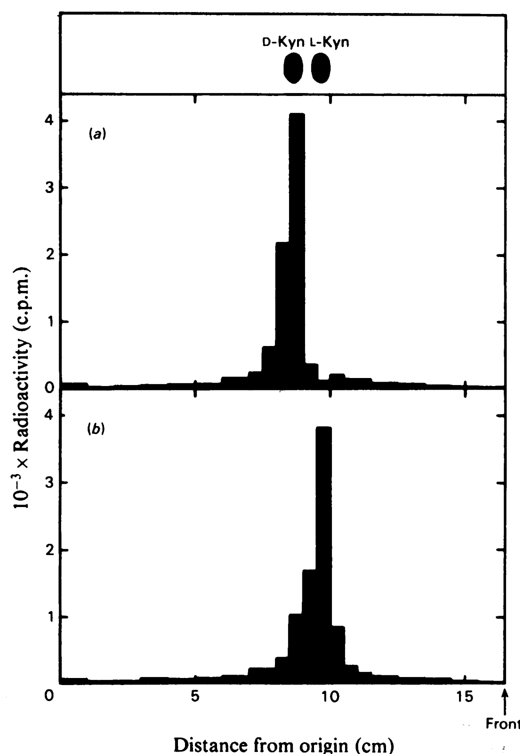

Fig. 5. *Identification of* D- *and* L-[^{14}C]*kynurenine by using cellulose t.l.c.*

The reaction mixture (500 μl) contained 50 mM-Hepes/NaOH, pH 7.3, 10 μM-Methylene Blue, 5.0 mM-ascorbate, 1.0 μM-catalase, 8.25 μg of formamidase, 25 mM-sodium formate, 4.68 μg of the purified enzyme after removal of L-tryptophan and CO, and D-[*benzene*-U-^{14}C]tryptophan (6.66×10^5 c.p.m., 1.5 μmol) or L-[*benzene*-U-^{14}C]tryptophan (5.54×10^5 c.p.m., 0.3 μmol). After incubation for 2 h at 25°C, the reaction was terminated by the addition of 200 μl of 0.5 M-HCl containing D- and L-kynurenine as carriers (2.5 μmol each) and then the solution was applied directly to a polyamide column (1.0 cm × 90 cm), previously equilibrated with 0.5 M-$BaCl_2$/0.1 M-HCl. The column was eluted with 100 ml of the same buffer and then with 30% (v/v) ethanol/0.1 M-HCl as described by Fujiwara *et al.* (1979). Fractions (2 ml) were collected. The kynurenine fractions (tubes 25–37) were combined and concentrated to approx. 5 ml under reduced pressure at 40°C. Then $BaCl_2$ was removed by the addition of 50 ml of ethanol followed by centrifugation at 10000 g for 10 min. The supernatant was concentrated to about 5 ml, lyophilized, dissolved in a small amount of water, and analysed by t.l.c. [^{14}C]Kynurenine produced from D-[*benzene*-U-^{14}C]tryptophan or L-[*benzene*-U-^{14}C]tryptophan (8200 c.p.m. and 8500 c.p.m. in 10 μl of water, respectively) was applied to cellulose t.l.c. plates (3 cm × 20 cm). The chromatogram was developed with a solvent system of pyridine/butan-1-ol/water (1:1:1, by vol.). The radioactivity was determined by scraping off the cellulose in 0.5-cm width strips followed by scintillation counting. (*a*) The product derived from D-[^{14}C]tryptophan; (*b*) the product derived from L-[^{14}C]tryptophan. Internal authentic D- and L-kynurenine were detected by their fluorescence under u.v. light. D-Kyn, D-kynurenine; L-Kyn, L-kynurenine.

D-isomer was also enhanced to the same extent in all cases. Injection of D-tryptophan increased both activities in a similar manner.

Purification of enzyme from mouse liver

The purification was performed as described in the Experimental section. The overall purification achieved was about 3000-fold with a yield of 2% (Table 2). Unless L-tryptophan and CO were used as stabilizers, both D- and L-tryptophan cleaving activities were extremely unstable and decreased especially at low protein concentrations and at low ionic strength. D-Tryptophan was much less effective as a stabilizer of the enzyme than the L-isomer. During all column chromatographies, only one peak of enzyme activity was observed. As shown in Table 2, the D-tryptophan cleaving activity was co-purified with the L-tryptophan cleaving activity, maintaining a constant ratio (0.26) throughout the purification. The final preparation, apparently homogeneous as judged by polyacrylamide-gel electrophoresis (Fig. 4*a*), had specific activities of 0.55 and 2.13 μmol/min per mg of protein at 25°C toward D- and L-tryptophan, respectively. The specific activity for L-tryptophan was comparable with that of tryptophan 2,3-dioxygenase from rat liver (Schimke, 1970; Schutz & Feigelson, 1972).

Identification of reaction products from D- *and* L-*tryptophan*

D- or L-[*benzene*-U-^{14}C]Tryptophan was incubated with the purified enzyme as described in the legend to Fig. 5. *N*-Formylkynurenine, the direct reaction product, was hydrolysed to kynurenine and formic acid by the action of the purified formamidase to facilitate the quantification and the stereospecific analysis of the reaction product. [^{14}C]Kynurenine derived from [*benzene*-U-^{14}C]tryptophan was separated from the substrate by using a polyamide column as described by Fujiwara *et al.* (1979). The recovery of internal authentic kynurenine throughout the column was estimated by measurement of A_{360} ($\varepsilon = 1.33$ mM$^{-1}\cdot$cm^{-1} in 0.1 M-HCl). The amount of [^{14}C]kynurenine at the termination of the reaction, corrected for recovery (approx. 85%), corresponded exactly to the amount of [^{14}C]formic acid derived from [*ring*-2-^{14}C]tryptophan under the same assay conditions. Control reactions with boiled enzyme did not produce any

radioactive substance in the kynurenine fractions of the column chromatography. The [^{14}C]kynurenine derived from D- and L-[*benzene*-U-^{14}C]tryptophan, as analysed by cellulose t.l.c., gave only a single radioactive peak that was located at the positions of D- and L-kynurenine respectively (Figs. 5*a* and 5*b*). These results indicate that the enzyme converts D-tryptophan to *N*-formyl-D-kynurenine and L-tryptophan to *N*-formyl-L-kynurenine, respectively. Similar results were obtained with the partially purified (step 3) enzyme from mouse liver.

Inactivation and inhibition of the enzyme

In order to verify that the D- and L-tryptophan cleaving activities of the apparently homogeneous enzyme were derived from a single protein, inactivation by heat and inhibition by various compounds were studied. After heat treatment for 15 min at different temperatures, the extent of inactivation of both activities was essentially the same, as shown in Fig. 6. Table 3 shows that both activities decreased to nearly the same extent when the reaction was carried out in the presence of various compounds, including thiol-specific reagents, KCN, NaN_3, and indole derivatives. KCN, NaN_3 (Tanaka & Knox, 1959) and indole derivatives (Frieden *et al.*, 1961) were reported to be inhibitors of rat liver tryptophan 2,3-dioxygenase. Although catalase, contained in the assay mixture as one of the activators, was reported to be inhibited by KCN and NaN_3 (Chance, 1952), the extent of inhibition of the dioxygenase activities was not altered in the presence of a 10-fold greater concentration of catalase. No inhibition by superoxide dismutase or Tiron (1,2-dihydroxybenzene-3,5-disulphonic acid) was observed with either activity of the purified enzyme, whereas indoleamine 2,3-dioxygenase has been reported to be inhibited by 70 and 75% in the presence of superoxide dismutase (6 μM) and Tiron (10 mM), respectively (Hirata & Hayaishi, 1975). All other attempts, including isoelectric focusing and gel electrophoresis, to separate the two components responsible for the two activities were unsuccessful. These results strongly suggest that a single enzyme degrades both stereoisomers, D- and L-tryptophan.

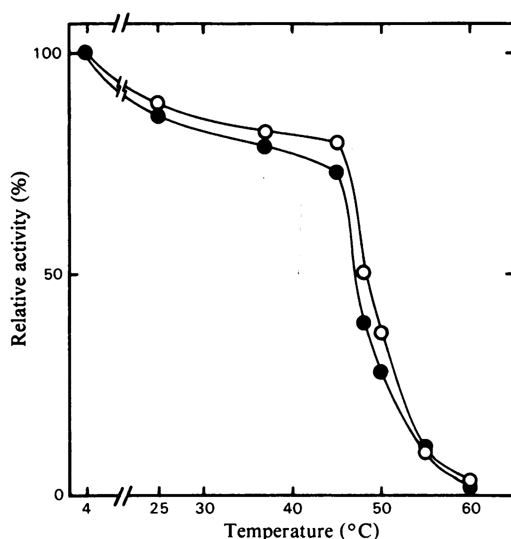

Fig. 6. *Heat inactivation of the purified enzyme*
After removal of L-tryptophan and CO by using a Sephadex G-25 column, heat treatment of the enzyme (2.5 μg) was performed at the temperatures indicated for 15 min. D- and L-tryptophan cleaving activities were measured by using procedure B at 25°C for 5 min. ●, D-Tryptophan cleaving activity; ○, L-tryptophan cleaving activity.

Kinetic studies on the active site

The nature of inhibition by the substrate analogue indole-3-acrylic acid, was investigated. This compound, a potent non-metabolizable inhibitor of rat liver tryptophan 2,3-dioxygenase (Frieden *et al.*, 1961), acted as a competitive inhibitor for both the D- and L-tryptophan cleaving activities of the purified enzyme from mouse liver (results not shown). The K_i values for indole-3-acrylic acid against the D- and L-tryptophan cleaving activities were essentially the same (6.8 and 4.5 μM, respectively). Further, the K_m values for D- and L-tryptophan (0.96 and 0.21 mM, respectively) agreed well with the K_i values for D-tryptophan against the L-tryptophan cleaving activity and for L-tryptophan against the D-tryptophan cleaving activity (0.9 and 0.2 mM, respectively) (Figs. 7*a* and 7*b*). These results indicate that a single active site is responsible for both activities.

Molecular and catalytic properties of the purified enzyme

The molecular weight of the enzyme was determined to be 150000 by gel filtration on Sephadex G-200. The molecular weights of the subunits were estimated to be 37000 and 40000 by SDS/polyacrylamide-gel electrophoresis (Fig. 4*b*). One mol of the enzyme contained 1.95 mol of protohaem IX as a prosthetic group, as well as 2.23 mol of iron and a trace amount (0.02 mol) of copper, as analysed by the procedure described in the Experimental section. These molecular properties appear to be similar to those of rat liver tryptophan 2,3-dioxygenase (Schutz & Feigelson, 1972). The optimum pH values of both activities of the purified enzyme were the same (pH 7.3). The substrate specificity of the purified enzyme was examined by measuring the increase in absorbance at each absorption maximum of the product corresponding to formylkynurenine (Shimizu *et al.*, 1978) at 25°C. Among a number of

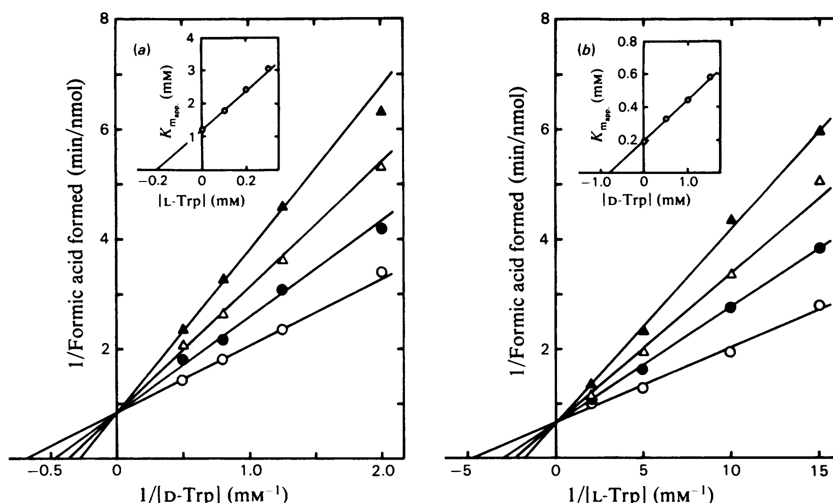

Fig. 7. *Competitive inhibition by* L- *and* D-*tryptophan respectively of* D- *and* L-*tryptophan cleaving activities*
Assays were performed at 25°C for 15 min by using procedure B as described in the Experimental section. Double reciprocal plots of the rates of the production of [^{14}C]formic acid against various concentrations of substrate (D- or L-[*ring*-2-^{14}C]tryptophan) in the presence of various concentrations of inhibitor (non-radioactive L- or D-tryptophan) are shown. (*a*) Substrate, D-[*ring*-2-^{14}C]tryptophan, inhibitor, non-radioactive L-tryptophan: ○, 0 mM; ●, 0.1 mM; △, 0.2 mM; ▲, 0.3 mM. Inset: replots of the results. (*b*) Substrate, L-[*ring*-2-^{14}C]tryptophan, inhibitor, non-radioactive D-tryptophan: ○, 0 mM; ●, 0.5 mM; △, 1.0 mM, ▲, 1.5 mM. Inset: replots of the results.

Table 4. *Stereospecificity of the purified tryptophan 2,3-dioxygenases from rat liver and Pseudomonas fluorescens*
The enzyme was passed through a column of Sephadex G-25 and assayed at 25°C by using procedure B with 2.0 mM-L-[*ring*-2-^{14}C]tryptophan (100 c.p.m./nmol) or 5.0 mM-D-[*ring*-2-^{14}C]tryptophan (300 c.p.m./nmol) as substrate under the standard assay conditions described in the Experimental section. The radioactivity obtained from control incubations with boiled enzyme (56 and 98 c.p.m. with L- and D-tryptophan as substrate respectively) has been subtracted from each value. Each value was obtained from duplicate experiments.

Enzyme source	Amount (μg)	Substrate	[^{14}C]Formate formed (c.p.m.)
Rat liver	0.9	L-Tryptophan	3150
	1.8	L-Tryptophan	6420
	1.8	D-Tryptophan	3820
	3.6	D-Tryptophan	6640
Pseudomonas fluorescens	0.32	L-Tryptophan	2340
	0.64	L-Tryptophan	3940
	0.64	D-Tryptophan	8
	1.28	D-Tryptophan	25

indoleamine derivatives tested, L-tryptophan gave the highest specific activity (2.13 μmol/min per mg of protein), followed by D-tryptophan (0.55 μmol/min per mg). No reaction was detected with 5-hydroxy-L- and 5-hydroxy-D-tryptophan, tryptamine, serotonin and indole-3-acetic acid although the amount of purified enzyme was 50 times that used with L-tryptophan.

Stereospecificity of the purified tryptophan 2,3-dioxygenases from rat liver and Pseudomonas

The highly purified enzymes from rat liver and *Pseudomonas fluorescens* were assayed with D- and L-[*ring*-2-^{14}C]tryptophan as substrates under the standard assay conditions described in the present paper. As shown in Table 4, the hepatic enzyme significantly acted on D-tryptophan. However, the bacterial enzyme did not act on the D-isomer.

Discussion

du Vigneaud *et al.* (1932) and Berg (1934) demonstrated that D-tryptophan could be utilized for growth of young rats as effectively as the L-isomer. This phenomenon has been interpreted to be due to the inversion of D-tryptophan to the L-isomer, via indolepyruvic acid, before further metabolism, evidence for which has been obtained in mammalian

liver and kidney slices by Kotake & Goto (1937, 1941). However, other studies on D-tryptophan metabolism indicated the direct conversion of D-tryptophan to D-kynurenine via *N*-formyl-D-kynurenine. Kotake Jr. & Ito (1937) isolated large amounts of D-kynurenine from the urine of rabbits fed D-tryptophan. Berg and co-workers obtained similar results with rats (Borchers *et al.*, 1942) and humans (Langner & Berg, 1955). Because the well-known hepatic tryptophan 2,3-dioxygenase was reported to be inactive with D-tryptophan by Knox and co-workers (Knox & Mehler, 1950; Tanaka & Knox, 1959; Civen & Knox, 1960), the enzyme responsible for conversion of D-tryptophan into D-kynurenine was sought and a D-tryptophan pyrrolase was isolated and partially purified from rabbit small intestine in this laboratory (Higuchi & Hayaishi, 1967; Yamamoto & Hayaishi, 1967). Since then, D-kynurenine in the urine has been considered to be solely derived from the action of this newly discovered enzyme, which is now referred to as indoleamine 2,3-dioxygenase because of its broad substrate specificity (Hirata *et al.*, 1974). Indoleamine 2,3-dioxygenase has been shown to be widely distributed in various tissues and organs of mammals, except in the liver (Hayaishi *et al.*, 1975). However, Berg and co-workers (Loh & Berg, 1971; Rodden & Berg, 1974) suggested the possible conversion of D-tryptophan into D-kynurenine using the crude and partially purified enzyme preparations from rat liver. Therefore, we examined the supernatant fraction of the liver of several species of mammals, which resulted in the finding of a significant D-tryptophan cleaving activity as well as an activity toward the L-isomer (Table 1). Since the conversion of D-tryptophan to the L-isomer by the supernatant fraction of the liver was reported by Rodden & Berg (1974) to be almost negligible, the activity toward D-tryptophan measured in the present study can be considered to represent the reaction rate of the direct conversion of D-tryptophan to *N*-formyl-D-kynurenine. Based on the results of the above screening test, the enzyme was purified from mouse liver to investigate the origin of the D-tryptophan cleaving activity in the liver.

The purified enzyme from mouse liver was similar to the tryptophan 2,3-dioxygenase purified from rat liver (Schimke, 1970; Schutz & Feigelson, 1972) and dissimilar to indoleamine 2,3-dioxygenase purified from mouse epididymis (K. Nakata, Y. Watanabe & O. Hayaishi, unpublished work) and rabbit small intestine (Shimizu *et al.*, 1978), with respect to molecular and catalytic properties. Further, inhibition by substrate or by scavengers of superoxide anion, both of which are characteristic of indoleamine 2,3-dioxygenase (Yamamoto & Hayaishi, 1967; Hirata & Hayaishi, 1975), was not observed with the mouse liver enzyme (Table 3). The data obtained in the present study established that a single enzyme, tryptophan 2,3-dioxygenase, catalyses both D- and L-tryptophan cleavages in the liver. The discrepancy concerning the stereospecificity of hepatic tryptophan 2,3-dioxygenase between the present results and the results of the previous investigators (Knox & Mehler, 1950; Tanaka & Knox, 1959; Civen & Knox, 1960; Schimke, 1970; Feigelson & Brady, 1974) may be explained by the following three reasons. They used (1) a much less sensitive method for the enzyme assay than our method with radioactive substrate, (2) rat liver as the enzyme source, in which the ratio of D- and L-tryptophan cleaving activities was quite low (0.07) even under our assay conditions (Table 1), and (3) ascorbate (around 0.5 mM) or a H_2O_2-generating system as an activator of the enzyme reaction. Under their assay conditions with either of these activators, the D-tryptophan cleavage catalysed by the purified enzyme from mouse liver would be specifically decreased (Figs. 1 and 2). From our preliminary spectral studies with mouse liver enzyme, destruction of the haem by an excess of H_2O_2 was observed in the presence of D-tryptophan but not in the presence of the L-isomer (Y. Watanabe & M. Sono, unpublished work). Therefore, one likely explanation for the apparent inactivation of the enzyme specific for D-tryptophan as substrate would be that L-tryptophan could prevent the enzyme from being inactivated by excess H_2O_2 under the assay conditions, whereas D-tryptophan could not show such a protective effect. The following three reports may support this interpretation. Civen & Knox (1960) first reported that D-tryptophan was much less effective than the L-isomer as a stabilizer of rat liver tryptophan 2,3-dioxygenase against heat. Schimke *et al.* (1965*a*) described similar results with heat, ethanol, urea and trypsin treatment. Knox & Piras (1967) also reported that the D-isomer promoted the conjugation of the apoenzyme from rat liver with haematin much less than did the L-isomer.

Under the assay conditions containing Methylene Blue, ascorbate and catalase, the enzyme appears to be fully activated. Brady *et al.* (1971) reported that reductive activation of aerobically aged rat liver tryptophan 2,3-dioxygenase (in the ferric form) by ascorbate was caused by H_2O_2 (and superoxide anion) generated by the auto-oxidation of ascorbate as well as by direct transfer of electrons from ascorbate to the enzyme. Under the present assay conditions, neither excess amounts of catalase nor scavengers of superoxide anion inhibited the enzyme reaction, indicating that H_2O_2 and superoxide anion are not involved in the activation of the enzyme by the above three activators. Therefore, the requirement for catalase can be explained by its protective effect against the inactivation of the enzyme caused

by excess H_2O_2. However, the role of Methylene Blue in the activation of the enzyme remains unknown and is currently under investigation.

We extend our gratitude to: Dr. Y. Ishimura and Dr. R. Makino of the Department of Biochemistry, School of Medicine, Keio University for pertinent advice and the gift of tryptophan 2,3-dioxygenases; Dr. Y. Tsuchida and Dr. K. Yugari of Central Research Laboratory, Ajinomoto Co., Inc. for analysis of the purity of D-tryptophan; Dr. T. Nukiwa of Jichi Medical School for participation in the early phase of this work; Dr. M. Nozaki, Dr. Y. Saeki, and Dr. A. Miyatake of Shiga University of Medical Science for the atomic absorption spectrophotometry; Dr. Y. Shizuta of Kochi Medical School and M. Ohara of Kyoto University of critical reading of the manuscript. This investigation was supported in part by a grant-in-aid for Scientific Research from the Ministry of Education, Science and Culture, Japan, and by research grant from the Intractable Diseases Division, Public Health Bureau, Ministry of Health and Welfare, Japan, the Naito Foundation, the Sakamoto Foundation, and Nippon Shinyaku Co.

References

Andrews, P. (1965) *Biochem. J.* **96**, 595–606
Berg, C. P. (1934) *J. Biol. Chem.* **104**, 373–384
Borchers, R., Berg, C. P. & Whitman, N. E. (1942) *J. Biol. Chem.* **145**, 657–666
Brady, F. O., Forman, H. J. & Feigelson, P. (1971) *J. Biol. Chem.* **246**, 7119–7124
Chance, B. (1952) *J. Biol. Chem.* **194**, 483–496
Civen, M. & Knox, W. E. (1960) *J. Biol. Chem.* **235**, 1716–1718
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
du Vigneaud, V., Sealock, R. R. & Van Etten, C. (1932) *J. Biol. Chem.* **98**, 565–575
Enzyme Nomenclature. Recommendations [1978] (1979) pp. 110–111, Academic Press, New York
Feigelson, P. & Brady, F. O. (1974) in *Molecular Mechanisms of Oxygen Activation* (Hayaishi, O., ed.), pp. 87–133, Academic Press, New York
Feigelson, P. & Greengard, O. (1961) *J. Biol. Chem.* **236**, 153–157
Frieden, E., Westmark, G. W. & Schor, J. M. (1961) *Arch. Biochem. Biophys.* **92**, 176–182
Fujiwara, M., Shibata, M., Nomiyama, Y., Sugimoto, T., Hirata, F., Tokuyama, T., Senoh, S. & Hayaishi, O. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 1145–1149
Gibson, Q. H. (1959) *Biochem. J.* **71**, 293–303
Hayaishi, O. & Stanier, R. Y. (1951) *J. Bacteriol.* **62**, 691–709
Hayaishi, O., Hirata, F., Fujiwara, M., Ohnishi, T. & Nukiwa, T. (1975) *Proc. FEBS Meet. 10th*, pp. 131–144, Federation of European Biochemical Societies, Amsterdam
Higuchi, K. & Hayaishi, O. (1967) *Arch. Biochem. Biophys.* **120**, 397–403
Hirata, F. & Hayaishi, O. (1971) *J. Biol. Chem.* **246**, 7825–7826
Hirata, F. & Hayaishi, O. (1972) *Biochem. Biophys. Res. Commun.* **47**, 1112–1119
Hirata, F. & Hayaishi, O. (1975) *J. Biol. Chem.* **250**, 5960–5966
Hirata, F., Hayaishi, O., Tokuyama, T. & Senoh, S. (1974) *J. Biol. Chem.* **249**, 1311–1313
Hirata, F., Ohnishi, T. & Hayaishi, O. (1977) *J. Biol. Chem.* **252**, 4637–4642
Ishimura, Y. (1970) *Methods Enzymol.* **17A**, 429–434
Knox, W. E. (1955*a*) *Methods Enzymol.* **2**, 242–246
Knox, W. E. (1955*b*) *Methods Enzymol.* **2**, 246–249
Knox, W. E. & Mehler, A. H. (1950) *J. Biol. Chem.* **187**, 419–430
Knox, W. E. & Ogata, M. (1965) *J. Biol. Chem.* **240**, 2216–2221
Knox, W. E. & Piras, M. M. (1967) *J. Biol. Chem.* **242**, 2959–2965
Kotake, Y. & Goto, S. (1937) *Hoppe-Seyler's Z. Physiol. Chem.* **248**, 41–56
Kotake, Y. & Goto, S. (1941) *Hoppe-Seyler's Z. Physiol. Chem.* **270**, 48–53
Kotake, Y. Jr. & Ito, N. (1937) *J. Biochem. (Tokyo)* **25**, 71–77
Langner, R. R. & Berg, C. P. (1955) *J. Biol. Chem.* **214**, 699–707
Loh, H. H. & Berg, C. P. (1971) *J. Nutr.* **101**, 1351–1358
Loh, H. H. & Berg, C. P. (1973) *J. Nutr.* **103**, 397–403
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
McCord, J. M. & Fridovich, I. (1969) *J. Biol. Chem.* **244**, 6049–6055
Ohnishi, T., Hirata, F. & Hayaishi, O. (1977) *J. Biol. Chem.* **252**, 4643–4647
Paul, K. G., Theorell, H. & Akeson, A. (1953) *Acta Chem. Scand.* **7**, 1284–1287
Peterkofsky, B. (1968) *Arch. Biochem. Biophys.* **128**, 637–645
Poillon, W. N., Maeno, H., Koike, K. & Feigelson, P. (1969) *J. Biol. Chem.* **244**, 3447–3456
Prescott, J. M., Schweigert, B. S., Lyman, C. M. & Kuiken, K. A. (1949) *J. Biol. Chem.* **178**, 727–732
Rodden, F. A. & Berg, C. P. (1974) *J. Nutr.* **104**, 227–238
Schimke, R. T. (1970) *Methods Enzymol.* **17A**, 421–428
Schimke, R. T., Sweeney, E. W. & Berlin, C. M. (1965*a*) *J. Biol. Chem.* **240**, 4609–4620
Schimke, R. T., Sweeney, E. W. & Berlin, C. M. (1965*b*) *J. Biol. Chem.* **240**, 322–331
Schutz, G. & Feigelson, P. (1972) *J. Biol. Chem.* **247**, 5327–5332
Shimizu, T., Nomiyama, S., Hirata, F. & Hayaishi, O. (1978) *J. Biol. Chem.* **253**, 4700–4706
Snell, E. E., Strong, F. M. & Peterson, W. H. (1937) *Biochem. J.* **31**, 1789–1799
Tanaka, T. & Knox, W. E. (1959) *J. Biol. Chem.* **234**, 1162–1170
Taniguchi, T., Hirata, F. & Hayaishi, O. (1977) *J. Biol. Chem.* **252**, 2774–2776
Taniguchi, T., Sono, M., Hirata, F., Hayaishi, O., Tamura, M., Hayashi, K., Iizuka, T. & Ishimura, Y. (1979) *J. Biol. Chem.* **254**, 3288–3294
Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
Yamamoto, S. & Hayaishi, O. (1967) *J. Biol. Chem.* **242**, 5260–5266