Biochem. J. (1975) **150**, 329–333
Printed in Great Britain

Stabilization of Rat Liver Tyrosine Aminotransferase by Tetracycline

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(*Received* 19 *February* 1975)

1. Rat liver tyrosine aminotransferase was purified 200-fold and an antiserum raised against it in rabbits. 2. Hepatic tyrosine aminotransferase activity was increased fourfold by tyrosine, twofold by tetracycline, 2.5-fold by cortisone 21-acetate and ninefold by a combination of tyrosine and cortisol administered intraperitoneally to rats. 3. Radioimmunoassay with ^{14}C-labelled tyrosine aminotransferase, in conjunction with rabbit antiserum against the enzyme, revealed that cortisol stimulates the synthesis of the enzyme *de novo*, but that tetracycline has no such effect. 4. Incubation of rat liver homogenates with purified tyrosine aminotransferase *in vitro* leads to a rapid inactivation of the enzyme, which tetracycline partially inhibits. 5. The inactivation is brought about by intact lysosomes, and the addition of 10mM-cysteine increases the rate of enzyme inactivation, which is further markedly increased by 10mM-Mg^{2+} and 10mM-ATP. Here again tetracycline partially inhibits the decay rate, leading to the inference that the increase of tyrosine aminotransferase activity *in vivo* by tetracycline is brought about by the latter inhibiting the lysosomal catheptic action.

Hepatic tyrosine aminotransferase (EC 2.6.1.5) activity is increased by a variety of hormones and pharmacologically unrelated drugs (Lin & Knox, 1957; Greengard & Baker, 1966; Civen *et al.*, 1967; Holten & Kenney, 1967; Granner *et al.*, 1968; Hager & Kenney, 1969; Pal *et al.*, 1969; Mukhtar & Krishna Murti, 1971). Although cortisol, glucagon, adrenaline and insulin are known to increase the rate of enzyme synthesis, the mechanism of increase of tyrosine aminotransferase activity by different drugs is still not unequivocally established. An earlier report (Mukhtar *et al.*, 1973) from this laboratory, based on the relative decay rate of tyrosine aminotransferase activity *in vitro* from normal and tetracycline-treated rats, suggests that the enhanced enzyme activity probably results from inhibition of its inactivation and degradation *in vivo*.

The present paper reports an investigation into the mechanism whereby tyrosine aminotransferase activity is increased by tetracycline. Radioimmunoassay data reveal that its degradation *in vivo* is inhibited. Data on inactivation *in vitro* support earlier inferences (Mukhtar *et al.*, 1973) that tetracycline stabilizes the enzyme by inhibition of its rate of degradation.

Materials and Methods

Chemicals

Analytical-reagent-grade sodium diethyldithiocarbamate, 2-oxoglutarate, $(NH_4)_2SO_4$, sucrose and potassium phosphate were purchased from BDH, Poole, Dorset, U.K.; tyrosine and EDTA were obtained from Eastman Kodak, Rochester, N.Y., U.S.A.; dithiothreitol and Carbowax were from Calbiochem, Los Angeles, Calif., U.S.A.; pyridoxal phosphate was from Merck, Darmstadt, Germany; cycloheximide was from Fluka A.G., Buchs, Switzerland; tetracycline was from Indian Drugs and Pharmaceuticals, New Delhi, India; Whatman DEAE-cellulose was from W. and R. Balston Ltd., Maidstone, Kent, U.K.; Bio-Gel P-150 was from Bio-Rad Laboratories, Richmond, Calif., U.S.A.; and ^{14}C-labelled algal protein hydrolysate was from Bhabha Atomic Research Centre, Trombay, Bombay, India.

Animals and injection procedure

Albino rats (Charles Foster strain), derived from the Central Drug Research Institute animal colony, and maintained on pellet diet (Hind Lever, Bombay, India), were used. In all cases the rats were starved for the 24h preceding the day of experiment, and, at 7:30 a.m., were injected intraperitoneally with the particular drug (in doses to be described below) in 2ml of 0.9% NaCl/100g body wt. (experimental), or 2ml of 0.9% NaCl/100g body wt. (control). The rats were killed 6h later by decapitation.

Preparation of liver cytosol

The liver was excised, rinsed in chilled (4°C) 0.9% NaCl, blotted, weighed, minced and homogenized in 4vol. of ice-cold 0.25M-sucrose in a glass Potter–Elvehjem homogenizer fitted with a motor-driven Teflon pestle. All operations were carried out at 4°C. The homogenates were centrifuged at 750*g* for 10min

to remove cell nuclei and debris, and finally centrifuged at 100000*g* for 1h. The supernatant was used as the enzyme source.

Tyrosine aminotransferase assay

The enzyme was assayed by the method of Diamondstone (1966). The assay system, in 3.1ml of 0.2M-potassium phosphate buffer, pH7.3, contained 19.2μmol of tyrosine, 0.12μmol of pyridoxal phosphate, 0.024μmol of sodium diethyldithiocarbamate, and a suitable portion of the enzyme preparation. The reaction was initiated by the addition of 0.3μmol of α-oxoglutarate, and terminated after 20min of incubation by the addition of 0.2ml of 10M-NaOH. The control tubes received α-oxoglutarate after the addition of NaOH, and the *p*-hydroxyphenylpyruvate formed was measured by following the increase in E_{331}. One enzyme unit was defined as the amount of enzyme required to catalyse the formation of 1μmol of *p*-hydroxyphenylpyruvate/min at 37°C.

Protein was measured by the method of Lowry *et al.* (1951), by using bovine serum albumin as the standard.

Purification of tyrosine aminotransferase

Tyrosine aminotransferase was induced in rats weighing between 150 and 200g by administration of a mixture of L-tyrosine (10mg) and cortisone 21-acetate (1.25mg) in 2ml of 0.9% NaCl/100g body wt. The animals received the injections at 7.30a.m. and were killed by decapitation at 1.30p.m. Their livers were excised, chilled and rinsed with cold 0.15M-KCl. By using the minced tissue as starting material, tyrosine aminotransferase was purified essentially by the procedure of Valeriote *et al.* (1969). The enzyme was concentrated by exosmosis of water with Carbowax instead of by freeze-drying; Bio-Gel P-150 was used in place of Sephadex G-200 for gel filtration, and the eluates were concentrated with the aid of Carbowax.

As shown in Table 1, the above procedure resulted in an over 200-fold purification of tyrosine aminotransferase. The enzyme preparation gave a single precipitin line with its antiserum in Ouchterlony (1958) gel plates and disc electrophoresis of the enzyme in polyacrylamide gel yielded three protein bands, as reported by earlier workers (Hyashi *et al.*, 1967).

Purification of lysosomes

Livers were homogenized in 10vol. of ethanolamine acetate buffer (pH7.4)–0.33M-sucrose, and centrifuged at 750*g* for 10min to remove cell nuclei and debris and again at 3300*g* for 10min to remove the heavy mitochondrial fraction. The supernatant was centrifuged at 16000*g* for 20min, at which value lysosomes and light mitochondria sedimented. This crude lysosomal fraction was further purified by washing once in the homogenizing medium and finally centrifuging (16000*g*) the sediment suspended in 0.33M-sucrose through a discontinuous sucrose density gradient (layer 1, 1.7ml of 0.7M-sucrose; layer 2, 1.5ml of 0.6M-sucrose; and layer 3, 1.3ml of 0.45M-sucrose). The pure and intact sedimented lysosomal fraction obtained was devoid of glucose 6-phosphatase (Shull *et al.*, 1956) and succinate dehydrogenase (Srikantan & Krishna Murti, 1955) activities. Specific activity of acid phosphatase (Nelson, 1966) of the lysosomal preparation was 514 units (μmol/min)/mg of protein.

Preparation of anti-(tyrosine aminotransferase) serum

Rabbits weighing between 1 and 1.5kg were injected with purified enzyme (3mg of enzyme protein) in an equal volume of complete Freund's adjuvant. The following schedule was used, involving one injection per week. In the first and second week, the enzyme preparation was injected into the foot pads of the animals. The subsequent injections were given intramuscularly in the thigh. In the fifth week, a booster dose of 6mg of the enzyme was administered intramuscularly without mixing with Freund's adjuvant. The animals were bled at intervals after the last injection and when the antiserum registered the highest titre, blood was collected, left for 1h at 37°C and then at 4°C overnight. The antiserum collected was centrifuged (2000*g*/10min) and the supernatant stored in the deep-freeze in small batches.

Table 1. *Purification of rat liver tyrosine aminotransferase*

Fraction	Protein (mg/ml)	Enzyme (units/ml)	Specific activity (units/mg of protein)	Purification (fold)
Whole homogenate	129.0	2655.0	20.5	1.0
$(NH_4)_2SO_4$ fraction	41.0	5477.5	133.6	6.5
Heat-treated fraction	16.2	8045.0	496.6	24.1
DEAE-cellulose eluate (concentrated)	18.8	49650.0	2640.9	128.8
Bio-Gel P-150 eluate (concentrated)	11.6	50000.0	4310.3	210.2

Administration of labelled protein hydrolysate

Carrier-free ^{14}C-labelled algal protein hydrolysate was injected in a single dose of 10μCi/100g body wt. intraperitoneally 1h before the rats were killed.

Immunoprecipitation of labelled tyrosine aminotransferase and counting procedure

Fractions containing tyrosine aminotransferase were incubated for 1h at 37°C with an equal volume of undiluted antiserum; this was sufficient to precipitate all the labelled tyrosine aminotransferase. The tubes were left at 4°C for 18h. The precipitate was collected by centrifugation (1500g/10min), washed three times with cold 0.9% NaCl and once with ether, dissolved in a minimal quantity of 0.1M-NaOH and applied on Whatman no. 3 filter-paper discs and placed in 10ml of scintillation fluid containing 0.4% 2,5-diphenyloxazole (PPO) and 0.1% 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (dimethyl-POPOP) in toluene, and counted for radioactivity in a Tracerlab liquid-scintillation counter at 90% counting efficiency. The supernatant layer was then subjected to a second identical immunoprecipitation with unlabelled enzyme (volume and enzyme units equivalent to labelled enzyme). The radioactive material obtained from this precipitation represents non-specific protein that co-precipitated with tyrosine and aminotransferase–antibody complex (Schimke *et al.*, 1965). The radioactivity thus counted, which constituted 15–20% of that in the first immunoprecipitation, was subtracted from the latter to correct partially for the radioactivity contributed by non-specific labelled proteins.

Tyrosine aminotransferase-inactivating system in vitro

The system consisted of about 200 tyrosine aminotransferase units in phosphate-buffered saline, pH7.4 (8.0g of NaCl, 0.2g of KCl, 1.15g of Na_2HPO_4, 0.2g of KH_2PO_4, 0.1g of $CaCl_2$ and 0.1g of $MgCl_2,6H_2O$ in 1 litre of water), crude lysosomes (1.2mg of protein) and 10mM-cysteine in a total volume of 5ml. The reaction mixture was incubated with gentle shaking at 37°C. Samples were removed at zero time, i.e. immediately after addition of lysosomes, and then every hour for a period of 4h, and assayed for enzyme activity. Under similar conditions, and by using purified lysosomes, the inactivation rate of the enzyme was also studied at pH5.0 in 0.05M-sodium citrate buffer–0.33M-sucrose.

Results

Stimulation of hepatic tyrosine aminotransferase

The effect of the drugs on hepatic tyrosine aminotransferase is shown in Table 2. The drugs promote increased enzyme activity differentially; per 100g body wt., 10mg of tyrosine brought about a fourfold increase; 10mg of tetracycline, a twofold increase; 1.25mg of cortisone 21-acetate a 2.5-fold increase and a combination of cortisol and tyrosine resulted in an almost ninefold increase.

Differential effect of cortisol and tetracycline on hepatic tyrosine aminotransferase

Tetracycline might bring about increased tyrosine aminotransferase activity either by stimulation of the rate of synthesis of the enzyme, or by inhibiting its rate of degradation. Results obtained by radioimmunoassay of the enzyme (Table 3) from normal rats and from tetracycline- or cortisol-treated rats show that whereas cortisol increased amino acid incorporation into tyrosine aminotransferase, thus

Table 2. *Differential stimulation by drugs in vivo of rat liver tyrosine aminotransferase*

Drug dose is per 100g body wt. Each value is the mean for three animals (duplicate assays) ± S.D.

Drug	Specific activity (units/mg of protein)
Control (150mM-NaCl)	3.20±0.9
Tyrosine (10mg)	11.88±1.1
Tetracycline (10mg)	5.71±1.8
Cortisol (1.25mg)	7.50±1.2
Cortisol (1.25mg)+tyrosine (10mg)	26.70±1.7

Table 3. *Differential effect of cortisone 21-acetate and tetracycline on tyrosine aminotransferase synthesis*

As described in the text, rats were injected intraperitoneally with the drug under test, and with ^{14}C-labelled algal protein hydrolysate. Tyrosine aminotransferase assays and measurements of enzyme radioactivity were made with liver cytosol preparations. Each value is the mean for three animals (duplicate assays) ± S.D.

Treatment	Body wt. (g)	Liver wt. (g)	Sp. activity of tyrosine aminotransferase (units/mg of protein)	Total tyrosine aminotransferase radioactivity (c.p.m./liver)
Control	30.0±5.0	0.95±0.27	3.52±1.0	6523±466
Cortisone 21-acetate	32.5±2.5	1.21±0.10	7.55±0.7	10817±971
Tetracycline	32.5±2.5	1.03±0.76	5.80±1.3	7384±386

Table 4. *Stabilizing effect of tetracycline in vivo on rat liver tyrosine aminotransferase*

Dose administered is per 100g body wt. Each value is the mean for three animals (duplicate assays) ± S.D.

Treatment	Specific activity (units/mg of protein)
Control	3.58 ± 0.5
Tetracycline (10mg)	5.85 ± 1.0
Cycloheximide (2mg)	2.73 ± 0.8
Tetracycline + cycloheximide (10mg) + (2mg)	4.89 ± 0.5

showing increased enzyme synthesis, tetracycline failed to show any such effect.

Stabilizing effect of tetracycline on tyrosine aminotransferase

Table 4 shows the effect of cycloheximide on the increase in hepatic tyrosine aminotransferase activity induced by tetracycline. The enzyme activity in rats treated with tetracycline and then cycloheximide was 1.5–2-fold higher than in rats receiving only cycloheximide. This compares with the nearly twofold increase in enzyme activity obtained when rats were injected with tetracycline compared with rats receiving only 0.9% NaCl. These results further support the earlier inference (Mukhtar *et al.*, 1973) that stimulation of enzyme activity by the drug is not due to increased enzyme synthesis, but is due to decreased turnover of the enzyme protein.

Effect of tetracycline on inactivation of hepatic tyrosine aminotransferase in vitro

On incubation of rat liver homogenates *in vitro* at 37°C, tyrosine aminotransferase activity decreases and is partially stabilized by tetracycline (Fig. 1). Addition of thiol reagents further increased the inactivation, but even under optimal conditions for inactivation (with 10mM-cysteine), tetracycline was still found to stabilize the enzyme *in vitro*. It was revealed that the decay of enzyme activity is brought about by lysosomes. In this system also (which included 10mM-cysteine), tetracycline was found to have a stabilizing effect on decay of the enzyme (Fig. 1).

Effect of Mg^{2+} and ATP on lysosomal inactivation of tyrosine aminotransferase

Tyrosine aminotransferase inactivation was also studied with purified lysosomes at pH 5.0. The initial rate of inactivation (over the first hour) was increased twofold with the addition of 10mM-cysteine and sixfold with the addition of $MgSO_4$ and ATP, each at 10mM.

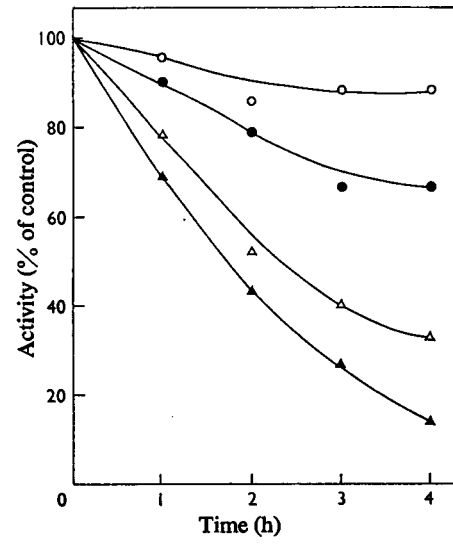

Fig. 1. *Effect of tetracycline on tyrosine aminotransferase inactivation in vitro*

Tyrosine aminotransferase was incubated with whole rat liver homogenate (●), homogenate plus 84 μM-tetracycline (○), lysosomes with cysteine (▲) or lysosomes with cysteine plus 84 μM-tetracycline (△) as described in the Materials and Methods section.

Discussion

Earlier studies from this laboratory (Mukhtar *et al.*, 1973) suggested that the stimulation of enzyme activity was brought about by stabilization of the enzyme *in vivo* by inhibiting its rate of degradation and thereby altering its half-life. Further observations on superinduction of the enzyme by the drug (Mukhtar *et al.*, 1973) supported these findings, and the mechanism of superinduction of the enzyme by actinomycin D (Reel & Kenney, 1968; Tomkins *et al.*, 1969) is still controversial.

Two experimental tools, namely the use of inhibitors and the radioimmunoassay of the enzyme, have been used for studying the effect of different agents on enzyme synthesis. The present studies thus further support our earlier observations (Mukhtar *et al.*, 1973) that tetracycline increases enzyme activity by increasing the enzyme content without stimulating the rate of enzyme synthesis.

Although in cortisol-treated rats there was increased incorporation of ^{14}C-labelled amino acids into tyrosine aminotransferase, tetracycline failed to enhance this incorporation. The higher enzyme activity in tetracycline-treated rats and in tetracycline+cycloheximide-treated rats compared with their corresponding controls would also support the stabilization hypothesis. Parallel studies on enzyme activity increases promoted by CCl_4 (Sato & Maruyana, 1974) and pyrimidine analogues (Cihak *et al.*, 1973) have also provided similar conclusions by the use of quantitative immunochemical precipitation and pulse-labelling techniques.

Inactivation of tyrosine aminotransferase was brought about by incubation with rat liver homogenates at 37°C. Further fractionation studies revealed that lysosomes were important constituents of the inactivating system. Cysteine stimulated the rate of inactivation by lysosomes. These observations are in agreement with the report of earlier workers (Auricchio *et al.*, 1972). Stimulation of tyrosine aminotransferase inactivation by Mg^{2+} and ATP suggests that requirements for the process are similar to those reported by Hyashi *et al.* (1973) for haemoglobin degradation. With the whole homogenate, and with lysosomes, tetracycline inhibited the rate of tyrosine aminotransferase decay. On the basis of these studies, it appears that the drug could either combine with the enzyme and make it refractory to lysosomal attack, or it may interfere with some crucial step involved in enzyme degradation.

We are grateful to Dr. C. R. Krishna Murti, Deputy Director and Head, Division of Biochemistry, for his encouragement, helpful suggestions and constructive criticism throughout the course of this investigation. This paper is Publication no. 2041 from the Central Drug Research Institute, Lucknow 226001, India. Part of this paper was presented at the 42nd Annual General Meeting of the Society of Biological Chemists, held at Mysore in November, 1973.

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