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TYROSINE TRANSAMINASE: INACTIVATION BY TYROSINE METABOLIC END PRODUCTS

MORTON CIVEN, CAROL WILSON, CHARLESTA B. BROWN
Medical Research Programs, Veterans Administration Hospital, Long Beach, Calif. (U.S.A.)

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SUMMARY

The tyrosine metabolites, *p*-hydroxyphenylpyruvate, homogentisate, and fumarylacetoacetate were examined as inhibitors and inactivators of purified rat liver tyrosine α-ketoglutarate transaminase. Of these, homogentisate proved to be a very powerful inactivator when the enzyme was preincubated with it. This effect was blocked by prior addition of pyridoxal phosphate, α-ketoglutarate and dithiothreitol, but could not be reversed by these compounds. The homogentisate inactivated enzyme retained its ability to interact with the antibody to native tyrosine α-ketoglutarate transaminase.

INTRODUCTION

The inducible enzyme, rat liver tyrosine-α-ketoglutarate transaminase (L-tyrosine:2-oxoglutarate aminotransferase, EC 2.6.1.5) has a half life of 2–3 h after induction[1,2]. Since end products of a metabolic pathway can inhibit the activity of an earlier enzymatic step, it was of interest to test for interaction between tyrosine–α-ketoglutarate transaminase and its metabolic end products in order to determine if this were a factor in the short half life of this enzyme. The interactions between tyrosine–α-ketoglutarate transaminase and the sequential degradation products of tyrosine, *p*-hydroxyphenylpyruvate, homogentisate, and fumarylacetoacetate, were studied to determine whether any of these compounds would inhibit tyrosine–α-ketoglutarate transaminase, or inactivate tyrosine–α-ketoglutarate transaminase when preincubated with the enzyme.

MATERIALS AND METHODS

Purified rat liver tyrosine α-ketoglutarate transaminase was prepared by the method of GRANNER *et al.*[3]. Fumarylacetoacetate was prepared enzymatically by incubating homogentisate with partially purified calf liver homogentisate oxidase and isolated from the reaction mixture as the silver salt according to the method of RAVDIN AND CRANDALL[4]. Homogentisate was purchased from Cyclo Chemical Co., Los Angeles, Calif.

Tyrosine–α-ketoglutarate transaminase activity was assayed by two methods. In the radioisotopic assay of WURTMAN AND LARIN[5] tyrosine α-ketoglutarate transaminase was incubated in a system containing [3,5-$^3H_2$]L-tyrosine (specific activity 47 C/mmole, Schwarz Bioresearch, Orangeburg, N.Y.). Tritiated *p*-hydroxyphenylpyruvate was isolated from the reaction mixture free of labeled tyrosine by solvent extraction. The activity of the *p*-hydroxyphenylpyruvate was measured in a liquid scintillation counter.

In the continuous spectrophotometric assay of LIN *et al.*[6] the rate of formation of the highly absorbent enol-borate complex of *p*-hydroxylphenylpyruvate was measured. This assay was not used to study the effect of fumarylacetoacetate since this compound absorbs in the same spectral region as the enol–borate complex of *p*-hydroxyphenylpyruvate. All incubations were carried out at 37°. The antigen–antibody reactions* between tyrosine α-ketoglutarate transaminase and its antibody were carried out according to the procedure of GRANNER *et al.*[3].

RESULTS

In the experiments reported here, *p*-hydroxyphenylpyruvate, homogentisate and fumarylacetoacetate were studied as inhibitors and as inactivators of tyrosine–α-ketoglutarate transaminase when the enzyme was preincubated with the compounds. Table I shows that direct addition of *p*-hydroxyphenylpyruvate, homogentisate and fumarylacetoacetate to the complete assay mixture inhibits only slightly tyrosine–α-ketoglutarate transaminase activity at the specified concentrations. Homogentisate when tested in a higher concentration range, between $1 \cdot 10^{-3}$ and $1 \cdot 10^{-2}$ M, produced a hyperbolic inhibition curve when tyrosine–α-ketoglutarate transaminase was assayed in the isotopic system. The inhibition is similar to that obtained in this assay system with serotonin or dopamine at the same concentrations.

When tyrosine–α-ketoglutarate transaminase was preincubated for 15 min at 37° with the test compounds, homogentisate and fumarylacetoacetate inactivated tyrosine–α-ketoglutarate transaminase, whereas *p*-hydroxyphenylpyruvate was ineffective. The final end product of the pathway, fumarylacetoacetate, is effective only at $3.3 \cdot 10^{-2}$ M. This concentration of fumarylacetoacetate is 1000 times greater than that necessary for homogentisate to produce the inactivation of tyrosine–α-ketoglutarate transaminase. Maleylacetoacetate, the compound intermediate between homogentisate and fumarylacetoacetate, was not studied since it could not be isolated free of contaminating homogentisate. The tyrosine–α-ketoglutarate transaminase preparations used, however, were shown to be free of homogentisate oxidase, the enzyme responsible for the conversion of homogentisate to maleylacetoacetate. Therefore, although the direct effects of maleylacetoacetate are not known, it is probable that the effects of homogentisate were not due to conversion to maleylacetoacetate.

Fig. 1 shows the kinetics of the inactivation process when tyrosine–α-ketoglutarate transaminase is incubated with homogentis at eatconcentrations of $6.7 \cdot 10^{-5}$ M, $3.3 \cdot 10^{-5}$ M and $1.68 \cdot 10^{-5}$ M. The inactivation of tyrosine–α-ketoglutarate trans-

* The antibody to tyrosine α-ketoglutarate transaminase was generously provided by Dr. Darryl Granner of the McArdle Laboratory, University of Wisconsin.

TABLE I

THE EFFECT OF TYROSINE METABOLITES ON TYROSINE–α-KETOGLUTARATE TRANSAMINASE ACTIVITY

Purified rat liver tyrosine–α-ketoglutarate transaminase (419 μmoles *p*-hydroxyphenylpyruvate formed per mg per h) was prepared at a concentration of 1.0 mg/ml in ovalbumin solution* (5 mg crystalline ovalbumin/ml). 50 μl of the tyrosine–α-ketoglutarate transaminase solution was mixed with either 40 μl of 0.1 M $NaPO_4$ (pH 7.6) or the above compounds dissolved in the buffer to give the specified final molarities in the preincubation mixture. The compounds and tyrosine–α-ketoglutarate transaminase were incubated 15 min at 37°. At this time the other components of the isotopic assay of tyrosine–α-ketoglutarate transaminase were added, and the incubation was continued for 20 min. The final concentrations of the test compounds in the tyrosine–α-ketoglutarate transaminase assay system were *p*-hydroxyphenylpyruvate, $1.0 \cdot 10^{-4}$ M; homogentisate, $1.0 \cdot 10^{-5}$ M; fumarylacetoacetate, $1 \cdot 10^{-2}$ M. The data, expressed as counts/min incorporated in the *p*-hydroxyphenylpyruvate per 20 min per 50 μl tyrosine–α-ketoglutarate transaminase, are the result of one experiment. Duplicate experiments gave qualitatively similar results.

Compounds	*15 min preincubation*	*Change (%)*	*No preincubation*	*Change (%)*
Control	828		1048	
p-Hydroxy phenylpyruvate ($3.3 \cdot 10^{-4}$ M)	813		1046	
Control	455		836	
Homogentisate ($3.3 \cdot 10^{-5}$ M)	39	−91.3	720	−13.8
Control	684		628	
Fumarylacetoacetate ($3.3 \cdot 10^{-2}$ M)	396	−42.2	625	0.0

* Although ovalbumin was used as a stabilizer of purified tyrosine–α-ketoglutarate transaminase[3], essentially similar results were obtained when the enzyme was dissolved in 0.1 M sodium phosphate (pH 7.4) without ovalbumin.

aminase by homogentisate at a concentration of $6.7 \cdot 10^{-5}$ M is extremely rapid, with 50% of the activity being degraded in about 60 sec. At homogentisate concentrations of $3.3 \cdot 10^{-4}$ M, tyrosine–α-ketoglutarate transaminase is completely inactivated in less than 30 sec.

The inactivation of tyrosine–α-ketoglutarate transaminase by homogentisate in the same concentration range was also observed using the continuous spectrophotometric assay. Linear tracings of absorbance against time were obtained with incubated controls, but in the tyrosine–α-ketoglutarate transaminase samples incubated with homogentisate, slight downward bowing was observed, indicating that inactivation still proceeds slowly, even after addition of the substrates and coenzyme of tyrosine–α-ketoglutarate transaminase.

Table II shows the effect of the preincubation of the individual components of the assay system on the homogentisate inactivation of tyrosine–α-ketoglutarate transaminase. In Part 1, tyrosine–α-ketoglutarate transaminase was first preincubated 5 min with either of the two substrates, and then homogentisate added, and the preincubation continued for another 5 min. Pyridoxal phosphate only partially blocked homogentisate's inactivating effect, while α-ketoglutarate completely blocked it. As can be seen from the control preincubations in Part 1, α-ketoglutarate and pyridoxal phosphate have opposing effects. The former activates tyrosine–α-ketoglutarate

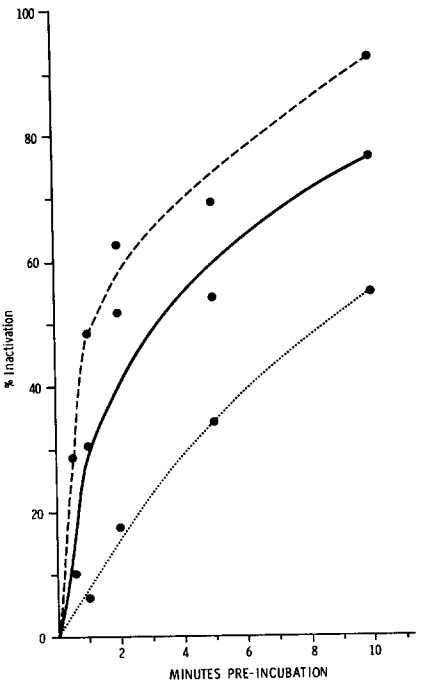


Fig. 1. Inactivation of purified rat liver tyrosine–α-ketoglutarate transaminase by three different concentrations of homogentisate. For incubation conditions see Table I. Homogentisate concn. (M): — — —, $6.7 \cdot 10^{-5}$; ———, $3.3 \cdot 10^{-5}$; · · · · ·, $1.7 \cdot 10^{-5}$.

transaminase whereas the latter inactivates it. These effects had been observed previously by LITWACK *et al.*[7] using a different tyrosine–α-ketoglutarate transaminase assay. Preincubation with 10^{-4} M dithiothreitol also blocked homogentisate's effect and activated tyrosine–α-ketoglutarate transaminase, but not to the same extent as α-ketoglutarate. Tyrosine had no protective effect when used in the amounts present in the assay. When the order of preincubation was reversed (Part 2) with the homogentisate added first, and preincubated 5 min, and the second component added with an additional 5-min preincubation, it can be seen that there was only slight reversal of the inactivation.

The variability in the controls in Part 2 is a reflection of the activating effect of α-ketoglutarate on the tyrosine–α-ketoglutarate transaminase activity. The much larger effect of homogentisate in Part 2 of the experiment is due to the fact that the total incubation period with homogentisate was twice as long as in Part 1 of the experiment. These results indicate that homogentisate irreversibly inactivates tyrosine–α-ketoglutarate transaminase.

The homogentisate-treated enzyme also could not be reactivated by 18-h dialysis at 4° against 0.05 M phosphate buffer (pH 7.0) containing 10^{-3} M versene. Examination of the absorption spectrum of the liquid in the dialysis bag showed that no detectable compound which absorbs in the same region as homogentisate was present.

When crude liver supernatant from triamcinolone-treated rats was used in

TABLE II

THE EFFECT OF DITHIOTHREITOL AND THE SUBSTRATE OF TYROSINE–α-KETOGLUTARATE TRANSAMINASE ON THE HOMOGENTISATE INACTIVATION OF TYROSINE–α-KETOGLUTARATE TRANSAMINASE WHEN ADDED EITHER BEFORE OR AFTER HOMOGENTISATE

Tyrosine α-ketoglutarate transaminase: ΔA per mg protein per 10 min. *Part 1*: Protective agents added before homogentisate. Tyrosine–α-ketoglutarate transaminase was preincubated for 5 min with dithiothreitol and with pyridoxal phosphate, α-ketoglutarate, or a combination of these two substrates. Homogentisate was added to give an incubation concentration of 886 μM and a final reaction concentration of 107 μM. Preincubation was continued for 5 min. *Part 2*: Protective agents added after homogentisate. Tyrosine–α-ketoglutarate transaminase was preincubated for 5 min with homogentisate concentration of 886 μM. Substrates or dithiothreitol, or a combination of the substrates, was added and preincubation continued for 5 min. Control samples were treated in the same manner as the experimental samples with the exception that buffer was substituted for homogentisate. On completion of both incubation periods, the remaining components of the continuous spectrophotometric assay were added, and the assay carried out, as described in MATERIALS AND METHODS.

Treatment	*Part 1**				*Part 2***			
	Incubation concn. (M)	*Control*	*Plus homogentisate*	*Change (%)*	*Incubation concn. (M)*	*Control*	*Plus homogentisate*	*Change (%)*
Base-line		11.8	7.5	−36.6		13.2	2.1	−84.0
Pyridoxal phosphate	$4 \cdot 10^{-4}$	7.5	6.3	−15.5	$3 \cdot 10^{-4}$	13.0	3.2	−75.2
α-Ketoglutarate	$2 \cdot 10^{-1}$	23.0	22.5	− 2.2	$2 \cdot 10^{-1}$	15.8	2.8	−82.4
Pyridoxal phosphate + α-ketoglutarate	$4 \cdot 10^{-4} + 2 \cdot 10^{-1}$	20.1	20.6	2.6	$3 \cdot 10^{-4} + 2 \cdot 10^{-1}$	20.6	3.5	−83.2
Dithiothreitol	10^{-4}	14.2	14.2	0.0	10^{-4}***	9.9	1.5	−84.4

* Average of two experiments.

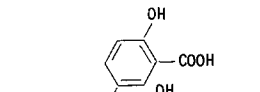
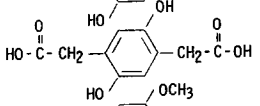
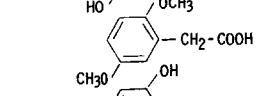
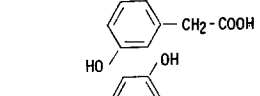
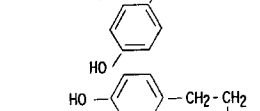
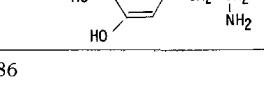
** Results of one experiment. Duplicate experiment at a different homogentisate concentration gave qualitatively the same results.

*** New enzyme solution. Therefore not the same base-line.

TABLE III

INACTIVATION OF TYROSINE–α-KETOGLUTARATE TRANSAMINASE BY COMPOUNDS STRUCTURALLY SIMILAR TO HOMOGENTISATE

Tyrosine–α-ketoglutarate transaminase was preincubated for 10 min at 37° with the compounds at a concentration of $1.68 \cdot 10^{-5}$ M.

	% INACTIVATION	
Gentisic Acid Sodium	21.8	
2, 5-dihydroxy-p-benzenediacetic acid	14.0	
(2, 5-dimethoxyphenyl) -acetic acid	12.2	
Homogentisic Acid	58.1	
Hydroquinone	86.3	
Dopamine	40.2	

place of the purified tyrosine–α-ketoglutarate transaminase preparation in the continuous spectrophotometric assay, inactivation of the tyrosine–α-ketoglutarate transaminase activity took place at $2 \cdot 10^{-3}$ M homogentisate, one thousand times the concentration required with the purified enzyme.

Table III shows the effects of preincubating tyrosine–α-ketoglutarate transaminase with compounds structurally related to homogentisate. Of those studied, only the ones which are readily oxidizable to the quinone form by atmospheric oxygen, hydroquinone, homogentisate and dopamine, showed significant inactivating ability. Hydroquinone was the most effective. Addition of the acetic acid side chain, as in homogentisate, lowered its effectiveness as an inactivator of tyrosine–α-ketoglutarate transaminase. Substitution of the acetic acid side chain by a carboxyl group as in gentisic acid markedly reduced the inactivating effect. Similarly, substitution of the ring hydroxyl groups by methoxy groups also reduced the inactivating effect. The addition of a second acetic acid side chain in a *p*-position to the first also blocked the inactivating effect.

In contrast to the results obtained with gentisic acid, TAKEMORI *et al.*[8] reported that gentisate is equally as effective as homogentisate in inhibiting bovine liver homogentisate oxidase.

Studies by CONSDEN *et al.*[9] show that at neutrality homogentisate is readily oxidized by atmospheric oxygen to benzoquinoneacetic acid. It was of interest to determine the effect of the addition of purified tyrosine–α-ketoglutarate transaminase on the rate of conversion of homogentisate to benzoquinoneacetic acid. The oxidation is readily followed spectrophotometrically since it is accompanied by marked alterations in the ultraviolet spectrum. The λ maximum for homogentisate is at 290 mμ ($\varepsilon = 3430$) while benzoquinoneacetic acid has its λ maximum at 250 mμ ($\varepsilon = 10\,790$) (ref. 9). Under our incubation conditions, (0.1 M sodium phosphate, pH 7.6, 37°), there was a rapid conversion of homogentisate to benzoquinoneacetic acid. This was followed by measuring the rate of increase in optical density at the trough in the homogentisate spectrum (270 mμ) which is close to the λ maximum of benzoquinoneacetic acid. The addition of ovalbumin to the media in the large concentrations used to stabilize the activity of purified tyrosine–α-ketoglutarate transaminase did not appreciably alter the rate of increase. In agreement, MASON AND PETERSON[10] had found that native ovalbumin does not react with indole-5, 6-quinone. However addition of purified tyrosine–α-ketoglutarate transaminase markedly inhibited the increase in $A_{270\ m\mu}$, and thus also the oxidative conversion of homogentisate to benzoquinoneacetic acid. This experiment indicates that purified tyrosine–α-ketoglutarate transaminase may be acting as an anti-oxidant in the homogentisate–benzoquinoneacetic acid system.

GRANNER *et al.*[3] found that when purified tyrosine–α-ketoglutarate transaminase was neutralized by its antibody, all catalytic activity was lost. If several different dilutions of antibody were added to purified tyrosine–α-ketoglutarate transaminase, the degree of antigen–antibody interaction could be measured quantitatively by assaying the reaction mixture for residual tyrosine–α-ketoglutarate transaminase activity. The plot of tyrosine–α-ketoglutarate transaminase activity remaining in the medium against the amount of anti-tyrosine–α-ketoglutarate transaminase antibody added, as shown in Fig. 2, is linear. If homogentisate-inactivated tyrosine–α-ketoglutarate transaminase is antigenically similar to native tyrosine–α-ketoglutarate

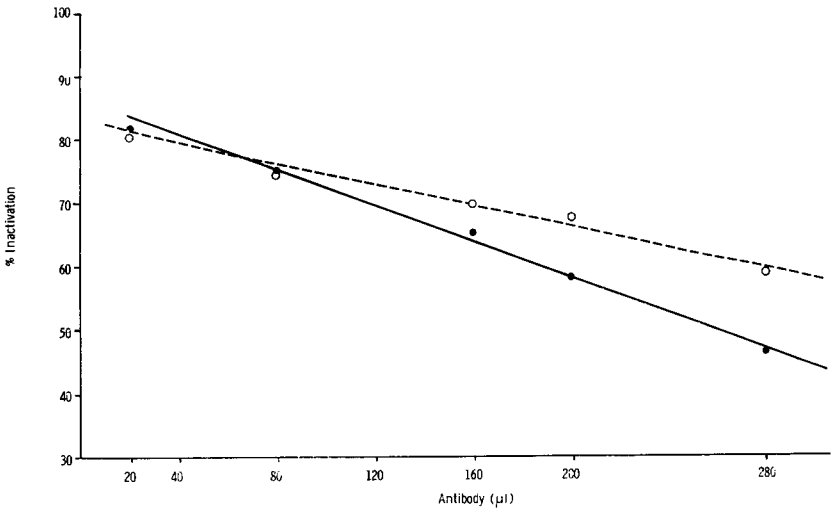


Fig. 2. Inactivation of tyrosine–α-ketoglutarate transaminase and homogentisate-inactivated tyrosine–α-ketoglutarate transaminase by antiserum to purified tyrosine–α-ketoglutarate transaminase. ———, native tyrosine–α-ketoglutarate transaminase; — — —, native tyrosine–α-ketoglutarate transaminase with homogentisate-inactivated tyrosine–α-ketoglutarate transaminase.

transaminase, it should compete for antibody binding sites. To determine this point, purified tyrosine–α-ketoglutarate transaminase was incubated with an amount of homogentisate ($1 \cdot 10^{-2}$ M) which produced complete inactivation. The inactivated enzyme preparation was then dialyzed exhaustively to remove homogentisate and its products and mixed with an equal amount of native tyrosine–α-ketoglutarate transaminase for immunoprecipitation studies. The dashed line in Fig. 2 shows this experiment. The decrease in slope of the inactivation curve indicates a decrease in the inactivation of native enzyme by its antibody due to competition between homogentisate inactivated and active tyrosine–α-ketoglutarate transaminase for the antibody. A similar observation was made by KENNEY[11] who found that tyrosine–α-ketoglutarate transaminase which was completely inactivated still fully retained its ability to react with its antibody.

DISCUSSION

The data obtained indicate that homogentisate produces an irreversible structural change in tyrosine–α-ketoglutarate transaminase which results in loss of activity. Thus in tyrosine's major metabolic pathway, it is not the final end product, fumarylacetoacetate, which is the most sensitive inhibitor of the first enzymatic step, but rather homogentisate, which is intermediate in the pathway. However, it should be added that another tyrosine metabolic pathway of some significance has been demonstrated in the rat[12]. This involves conversion of *p*-hydroxyphenylpyruvate to *p*-coumaric, *p*-hydroxybenzoic, and phloretic acids. These compounds were not tested as tyrosine–α-ketoglutarate transaminase inhibitors and their role in the metabolic control of this pathway remains to be assessed.

Pretreatment of tyrosine–α-ketoglutarate transaminase with either α-ketoglutarate, or dithiothreitol completely blocked homogentisate's inactivating effect, while pyridoxal phosphate only partially blocked inactivation. These compounds have all been shown to be general protective agents, useful in the purification of tyrosine–α-ketoglutarate transaminase[3] and glutamic-oxalacetic transaminase[13]. On the other hand, when homogentisate is added prior to the addition of these protective agents, the inactivating effect of homogentisate on tyrosine–α-ketoglutarate transaminase is not reversed. This might indicate that if the enzyme is in its fully active configuration, the group or groups vulnerable to homogentisate attack are not exposed. However, if the enzyme is attacked by homogentisate or its quinone derivative, it can no longer assume the active configuration.

Our spectral studies and those of CONSDEN *et al.*[9] indicate that homogentisate is rapidly converted to benzoquinoneacetic acid. The enzyme, tyrosine–α-ketoglutarate transaminase, but not large amounts of ovalbumin, is able to block the oxidative conversion. This indicates a structural relationship may exist between homogentisate and tyrosine–α-ketoglutarate transaminase.

Studies with compounds structurally similar to homogentisate (Table III) demonstrate the necessity of the aromatic hydroquinone structure for the interaction. Nevertheless, absolute specificity was not found since hydroquinone is even more effective than homogentisate. However, many instances are known where structural analogues of naturally occurring inhibitors or inducers are more effective than naturally occurring compounds. It has also been found that glutamate–oxalacetate transaminase from red blood cells was only inhibited 15% after preincubation with 10^{-4} M homogentisate*. This also points to protein specificity in the homogentisate–tyrosine–α-ketoglutarate transaminase interaction.

Although there is no direct evidence that homogentisate is involved in the *in vivo* regulation of tyrosine–α-ketoglutarate transaminase activity, certain observations in the literature suggest that it may play a role. KNOX AND GOSWAMI[14] found that after oral tyrosine administration to normal guinea pigs, liver tyrosine–α-ketoglutarate transaminase activity increased 3-fold, while *p*-hydroxyphenylpyruvate oxidase, the enzyme responsible for homogentisate formation fell to 18% of its normal level. The maximal depression of *p*-hydroxyphenylpyruvate oxidase is reached very rapidly and precedes the maximal elevation of tyrosine–α-ketoglutarate transaminase by 20 h. Tyrosine–α-ketoglutarate transaminase only falls from its high point significantly after *p*-hydroxyphenylpyruvate oxidase has returned to normal. LIN AND KNOX[15] also observed that while cortisol induction of tyrosine–α-ketoglutarate transaminase produced a 4-fold increase in this enzyme, there was also a small (24%) but significant increase in homogentisate oxidase, the enzyme which degrades homogentisate, whereas *p*-hydroxyphenylpyruvate oxidase, the enzyme which forms homogentisate, remains constant. The two induction curves closely paralleled each other in time. KNOX AND GOSWAMI[14] found that when 5-week-old rats were fed on a diet containing 5% L-tyrosine for a period of 5 weeks, tyrosine–α-ketoglutarate transaminase activity fell 30%. During this period homogentisate excretion increased from unmeasurable levels to 500 μmoles homogentisate per rat per day. By the fourteenth week, homogentisate excretion had fallen to insignificant levels, and tyrosine–α-

* These determinations were kindly carried out by Dr. Imre Fischer.

ketoglutarate transaminase once more rose to slightly higher levels than found at 5 weeks. These studies then point to an inverse correlation between tyrosine–α-ketoglutarate transaminase activity and homogentisate concentration. It will be of interest to further assess the significance of homogentisate in controlling tyrosine–α-ketoglutarate transaminase activity by determining the tissue levels of homogentisate as well as the enzymes responsible for its formation and degradation in relation to the changes in tyrosine–α-ketoglutarate transaminase activity following hormonal induction by agents such as glucagon where the fall in tyrosine–α-ketoglutarate transaminase activity after induction is more rapid than after glucocorticoids.

REFERENCES

1 L. GOLDSTEIN, E. J. STELLA AND W. E. KNOX, *J. Biol. Chem.*, 237 (1962) 1723.
2 F. T. KENNEY, *J. Biol. Chem.*, 237 (1962) 3495.
3 D. K. GRANNER, S. I. HAYASHI, E. B. THOMPSON AND G. M. TOMKINS, *J. Mol. Biol.*, 35 (1968) 291.
4 R. G. RAVDIN AND D. I. CRANDALL, *J. Biol. Chem.*, 189 (1951) 137.
5 R. J. WURTMAN AND F. LARIN, *Biochem. Pharmacol.*, 17 (1968) 817.
6 E. C. C. LIN, B. M. PITT, M. CIVEN AND W. E. KNOX, *J. Biol. Chem.*, 233 (1958) 668.
7 G. LITWACK, I. WINICOV AND J. M. SQUIRES, *Biochim. Biophys. Acta*, 128 (1966) 404.
8 S. TAKEMORI, E. FURUYA, K. MIHARA AND M. KATAGIRI, *European J. Biochem.*, 6 (1968) 411.
9 R. CONSDEN, H. A. W. FORBES, L. E. GLYNN AND W. M. STANIER, *Biochem. J.*, 50 (1951) 274.
10 H. W. MASON AND E. W. PETERSON, *Biochim. Biophys. Acta*, 111 (1965) 134.
11 F. T. KENNEY, *J. Biol. Chem.*, 237 (1962) 1605.
12 A. N. BOOTH, M. S. MASTI, D. J. EMERSON, H. OLIVER, F. T. JONES AND F. DEEDS, *J. Biol. Chem.*, 235 (1960) 2649.
13 W. T. JENKINS, D. A. YPHANTIS AND I. W. SIZER, *J. Biol. Chem.*, 234 (1959) 51.
14 W. E. KNOX AND M. N. D. GOSWAMI, *J. Biol. Chem.*, 235 (1960) 2662.
15 E. C. C. LIN AND W. E. KNOX, *J. Biol. Chem.*, 233 (1958) 1186.