Biochem. J. (1971) **125**, 563–568
Printed in Great Britain

The Inactivation of Phenylalanine Hydroxylase by 2-Amino-4-hydroxy-6,7-dimethyltetrahydropteridine and the Aerobic Oxidation of the Latter

THE EFFECTS OF CATALASE, DITHIOTHREITOL AND REDUCED NICOTINAMIDE–ADENINE DINUCLEOTIDE

BY A. JAKUBOVIČ, L. I. WOOLF AND E. CHAN-HENRY
Kinsmen Laboratory of Neurological Research, Department of Psychiatry, University of British Columbia, Vancouver, B.C., Canada

(*Received* 28 *June* 1971)

1. Phenylalanine hydroxylase is inhibited by its cofactor, 6,7-dimethyltetrahydropterin. The rate of inactivation, which is irreversible, increases with the concentration of cofactor. 2. Catalase, in sufficient amount relative to cofactor, prevents this inactivation. More tyrosine is formed in the presence of added catalase. 3. Dithiothreitol in the presence of liver extract also prevents inactivation of the enzyme by the cofactor and stimulates hydroxylation of phenylalanine, probably by protecting the cofactor from oxidation and regenerating it from a dihydropterin reaction product. Dithiothreitol restores linearity of rate at very low enzyme concentrations. 4. Dimethyltetrahydropterin is unstable when the solution is exposed to air but is stabilized by dithiothreitol the aerobic oxidation of which is greatly accelerated by dimethyltetrahydropterin. 5. NADH together with liver extract stabilizes the cofactor but not phenylalanine hydroxylase. 6. It is suggested that either hydrogen peroxide or an organic peroxide formed by oxidation in air of the cofactor is the substance attacking phenylalanine hydroxylase, dithiothreitol and cofactor.

It has been reported that high concentrations of 2-amino-4-hydroxy-6,7-dimethyltetrahydropteridine, a cofactor for the enzymic hydroxylation of phenylalanine to tyrosine, inhibit phenylalanine hydroxylase, the relevant enzyme (Kaufman, 1962; Bublitz, 1969; Zannoni & Moraru, 1970; Woolf, Jakubovič, Woolf & Bory, 1970). Kaufman (1962) suggested that hydrogen peroxide was formed and inactivated the enzyme and cofactor; catalase had a protective effect (Kaufman, 1962, 1970). Bublitz (1969) confirmed this finding and reported that thiol compounds stimulated the overall reaction by regenerating the cofactor (cf. Kaufman, 1959). Zannoni & Moraru (1970) suggested that the inhibitory effect of cofactor was a competitive inhibition in a multisubstrate–multiproduct reaction. We report here the rate of inactivation of phenylalanine hydroxylase in liver extracts by excess of the cofactor and the protection afforded by catalase and dithiothreitol, as well as the interactions of 6,7-dimethyltetrahydropterin and dithiothreitol in the presence of oxygen.

MATERIALS

L-Phenylalanine (Grade A) was purchased from Calbiochem (Los Angeles, Calif., U.S.A.) and recrystallized twice from ethanol–ammonia–water before use. 6,7-Dimethyl-5,6,7,8-tetrahydropterin, NADH and dithiothreitol were used as purchased from Calbiochem. α-Nitroso-β-naphthol (reagent grade) was obtained from Fisher Scientific Co. (Fair Lawn, N.J., U.S.A.) and recrystallized once from light petroleum (b.p. range 100–120°C) and once from ethanol before use. Catalase (ox liver, crystalline, suspension 183000 units/ml) and peroxidase (horseradish, 3075 units/mg) were purchased from Worthington Biochemical Corp. (Freehold, N.J., U.S.A.).

METHODS

Adult Wistar rats or C57 Bl mice were killed by decapitation and the livers immediately removed and homogenized in 4, 14 or 28 vol. of cold 0.15M-KCl. The 1:4 (w/v) homogenates were frozen and stored at −65°C; the more dilute homogenates were immediately centrifuged at 27000*g* for 30 min and the supernatants incubated with phenylalanine as shown in the Tables and Figures.

Protein in the liver supernatant was measured by the method of Warburg & Christian (1941).

Immediately after the reaction the tyrosine was determined in a sample of the deproteinized supernatant (Woolf *et al.* 1970) by Phillips' (1967) modification of the method of Waalkes & Udenfriend (1957), by using an Aminco–Bowman spectrofluorimeter at an exciting wavelength of 465nm and measuring the emission at 550nm. In each experiment all values were corrected for a control blank that contained the appropriate liver extract boiled for 3min and incubated under the same conditions; hence the contributions of the tyrosine contained in the liver and that formed non-enzymically during the incubation were eliminated from the determination.

RESULTS

Effect of dimethyltetrahydropterin and catalase. Hydroxylation of phenylalanine with fresh mouse liver supernatant (Fig. 1) was inhibited if the concentration of added cofactor was higher than 0.25mM; about 80% less tyrosine was formed with 2.0mM- than with 0.1mM-dimethyltetrahydropterin. Addition of catalase, however, prevented this inhibition. Fig. 2 shows that when rat liver supernatant prepared from frozen homogenate (freezing partly destroys catalase) was used, even 29 units of added catalase had a marked stimulatory effect on phenylalanine hydroxylation. Only in the presence of sufficient amounts of catalase is there a direct relationship between the amount of tyrosine formed and the concentration of the cofactor. The effect of time of incubation on the amount of tyrosine formed has been reported elsewhere (Woolf *et al.* 1970).

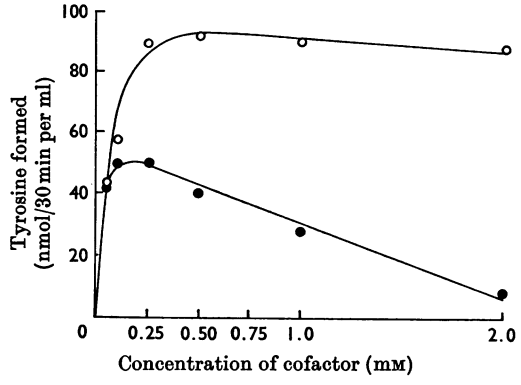

Fig. 1. Effect of varying the concentration of dimethyltetrahydropterin, with and without added catalase, on tyrosine formation by mouse liver supernatant. Incubation mixture: phenylalanine (2μmol), supernatant of the 1:28 mouse liver homogenate (0.1ml, 0.277mg of protein), NADH (0.8μmol), nicotinamide (5μmol), 0.2M-phosphate buffer, pH6.8, to 1ml; cofactor and catalase as indicated. The incubation was for 30min at 25°C, with shaking in air. ●, Without catalase; ○, with catalase (4100 units).

Effect of dithiothreitol. The results in Table 1 indicate that, in the presence of an appropriate concentration of dithiothreitol, very nearly the same amount of tyrosine was formed with mouse liver supernatant as when catalase was added. With low concentrations of phenylalanine hydroxylase, i.e. small volumes of liver supernatant, the rate of tyrosine formation decreased more rapidly than the enzyme concentration. However, addition of dithiothreitol restored the linearity at these low enzyme concentrations (Fig. 3). No inhibition by dithiothreitol, even 40mM, was apparent under the conditions used in these experiments.

Inactivation of phenylalanine hydroxylase by dimethyltetrahydropterin. Preincubation of the supernatant from frozen liver homogenate with dimethyltetrahydropterin and NADH for various times before addition of phenylalanine and catalase inactivated the phenylalanine hydroxylase at a measurable rate (Table 2). The results could not be attributed to destruction of the cofactor since addition of more liver extract with the phenylalanine and catalase caused the expected formation of tyrosine from phenylalanine. Where catalase (1830 units/ml) or dithiothreitol (5mM) was present during preincubation, the enzyme was completely protected. An amount of peroxidase equivalent in terms of haem groups to the catalase used had only a very small effect on tyrosine formation. Phenylalanine hydroxylase was stable after 45min of preincubation in the absence of cofactor.

Effect of hydrogen peroxide. Preincubation of liver supernatant (prepared from frozen homogenate to decrease the content of catalase activity) with hydrogen peroxide greatly decreased the rate of tyrosine formation (Table 3). Addition of catalase before hydrogen peroxide, as expected, had a protective effect; however, if catalase was added 5min after the hydrogen peroxide, about 35% less tyrosine was formed in the incubation. Dithiothreitol, even if added before hydrogen peroxide, was much less effective than catalase, though more tyrosine was formed than with the control. Most tyrosine was formed in the presence of both catalase and dithiothreitol when catalase was added before the hydrogen peroxide.

Stability of dimethyltetrahydropterin in solution. The stability of dimethyltetrahydropterin dissolved in phosphate buffer, pH6.8, was studied. The results in Table 4 show that after 30 and 45min of preincubation at 25°C in air about 70% and 100% respectively of the cofactor activity was lost; later addition of catalase and/or dithiothreitol to the incubation mixture was without effect. The rate of destruction was the same in darkness or diffused

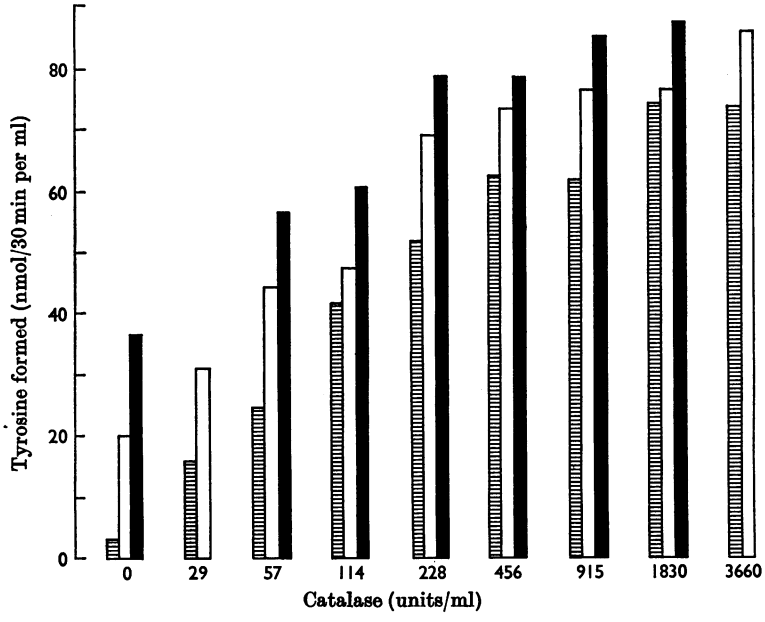

Fig. 2. Effect of varying the concentration of catalase on phenylalanine hydroxylation by rat liver supernatant with different amounts of dimethyltetrahydropterin. The incubation mixture was as described in Fig. 1; the amount of catalase was as indicated; the amount of cofactor was 0.5 (■), 1.0 (⊓) or 1.5 (目) μmol. The incubation was for 30 min at 25°C, with shaking in air.

Table 1. *Effect of catalase and dithiothreitol on phenylalanine hydroxylation by mouse liver supernatant*

The standard incubation procedure was used (see Fig. 1), but with dimethyltetrahydropterin 1.0 μmol, 0.1 ml of mouse liver supernatant (1:28 homogenate; 0.326 mg of protein), catalase and/or dithiothreitol added as indicated, in a total volume of 1 ml. Incubation was for 30 min.

Addition		
Catalase (units)	Dithiothreitol (μmol)	Tyrosine formed (nmol)
0	0	20.8
4100	0	83.3
4100	20	87.5
0	20	87.5
4100	10	95.8
0	10	95.8

sunlight, but dimethyltetrahydropterin was more stable at 0°C.

If dithiothreitol was present from the start of preincubation, dimethyltetrahydropterin remained fully active even after 45 min of preincubation (Table 5), but catalase under the same conditions was ineffective in preserving the cofactor. NADH and liver supernatant together, but not separately, had an effect similar to that of dithiothreitol (Table 5). As measured by the extinction at 360 nm, virtually no NADH was oxidized by incubation with dimethyltetrahydropterin but without liver supernatant.

Oxidation of dithiothreitol by air in phosphate buffer at 25°C is relatively slow (60% in 24 h); however, with cofactor there is a rapid oxidation of the former (Fig. 4). The concentration of cofactor remained constant until all the dithiothreitol had been oxidized and then decreased rapidly (Fig. 4). The presence or absence of phenylalanine, NADH or nicotinamide did not affect this. At higher concentrations of dimethyltetrahydropterin, the oxidation of dithiothreitol was more rapid. Dithiothreitol was determined with Ellman's (1959) reagent; cofactor was determined in a suitable sample with rat liver supernatant under the conditions of the standard assay. The same incubation mixture, but with different known concentrations of dimethyltetrahydropterin, gave a standard curve.

DISCUSSION

The results show that phenylalanine hydroxylase is progressively inactivated by its cofactor. This effect of dimethyltetrahydropterin is very fast and

most of the inactivation of the enzyme occurs in the first few minutes after the cofactor is added to the incubation mixture. Added hydrogen peroxide has a similar effect (Table 3). A direct relation between the rate of tyrosine formation and the initial concentration of cofactor is observed only if sufficient catalase is present (Figs. 1 and 2). The protective effect of catalase suggests that the inactivation of phenylalanine hydroxylase may be mediated by hydrogen peroxide formed by reaction between dimethyltetrahydropterin and oxygen (Kaufman, 1962), however the presence of organic peroxides that inactivate phenylalanine hydroxylase cannot be excluded.

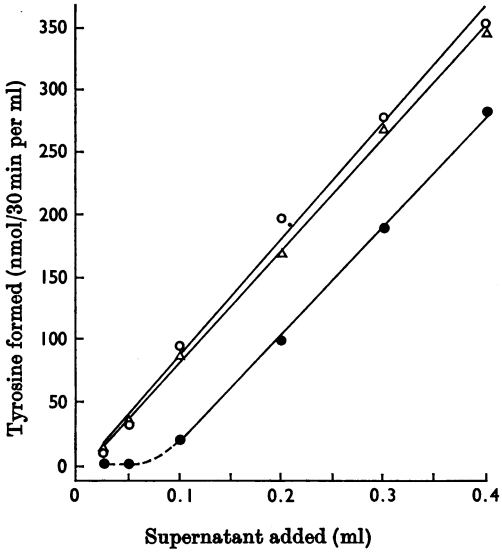

Fig. 3. Effect of varying the amount of enzyme and of the addition of dithiothreitol on tyrosine formation. The incubation mixture was as described in Fig. 1 but with 1.0 mM-cofactor and various volumes of mouse liver supernatant (1:28 homogenate)/ml; 0.260 mg of protein in 0.1 ml of liver supernatant. The incubation was for 30 min at 25°C, with shaking in air. ●, No dithiothreitol; ○, 5 mM-dithiothreitol; △, 40 mM-dithiothreitol.

Dithiothreitol has an ability to stimulate the rate of tyrosine formation in the presence of cofactor very similar to that of catalase, but some catalase activity was always present in our mouse or rat liver supernatant. Dithiothreitol may act in several ways: as a very effective reducing agent it regenerates the active cofactor from the quinonoid dihydropterin formed in the hydroxylation of phenylalanine (Bublitz, 1969; Jakubovič, 1971) and also protects the cofactor from rapid oxidation by air (Fig. 4, Table 5), hence sufficient active cofactor is present throughout the reaction so long as there is excess of dithiothreitol. The lack of inhibitory effect of high concentrations of dithiothreitol on tyrosine formation with mouse and rat liver supernatant differs from that found with purified rat liver phenylalanine hydroxylase (Bublitz, 1969), but is in agreement with results with human foetal-liver extracts (Jakubovič, 1971).

The oxidation of dimethyltetrahydropterin dissolved in phosphate buffer to the inactive non-reducible dihydropterin is a fast reaction (Table 4), the mechanism of which has been discussed by Kaufman (1964) and by Viscontini, Leidner, Mattern & Okada (1966). As shown in Table 5, the inactivation of cofactor is prevented by dithiothreitol. Catalase, although protecting the phenylalanine hydroxylase, can neither prevent the in-

Table 2. *Inactivation of phenylalanine hydroxylase on preincubation with dimethyltetrahydropterin*

Rat liver supernatant (0.1 ml of the 1:28 homogenate) was preincubated for various times at 25°C in air with dimethyltetrahydropterin (0.3 μmol), NADH (0.8 μmol), nicotinamide (5 μmol) and 0.2 M-sodium phosphate buffer, pH 6.8, to a total vol. of 0.5 ml. After preincubation, 2 μmol of phenylalanine was added to each tube and further additions were made as shown. The volume was adjusted to 1 ml and incubation continued for a further 20 min, then tyrosine was determined.

	Addition at incubation stage				Tyrosine formed (nmol)				
Preincubation time (min) ...	Catalase (units)	Dithiothreitol (μmol)	Cofactor (μmol)	Liver supernatant (ml)	0	3	5	10	45
	0	5	0.1	0	23.6	13.4	8.4	7.3	1.1
	1830	0	0.1	0	15.1	14.6	14.0	7.8	1.1
	1830	0	0	0.1	41.7	41.7	39.4	30.3	22.5
	0	0	0.1	0	—	—	—	—	27.8*
	0	0	0.1	0	—	—	—	—	21.0†

* Dithiothreitol (5 μmol) was present throughout 45 min of preincubation.
† Catalase (1830 units) was present throughout 45 min of preincubation.

Table 3. *Effect of hydrogen peroxide on phenylalanine hydroxylation by rat liver: modification by catalase and dithiothreitol*

To the supernatant from a 1:28 homogenate of frozen rat liver (0.1 ml, 0.499 mg of protein) was added either (A) 1.0 or (B) 2.0 μmol of H_2O_2 in a total volume of 1 ml of 0.2 M-sodium phosphate buffer, pH 6.8. Catalase (1830 units) and/or dithiothreitol (10 μmol) was added at various times as shown; where zero time of addition is shown, catalase or dithiothreitol was added before H_2O_2. At 2 min after the final addition (12 min in the first tube), phenylalanine (2.0 μmol), cofactor (0.3 μmol), NADH (0.8 μmol) and nicotinamide (5 μmol) were added to each tube and the mixtures incubated for 30 min at 25°C.

	Catalase			Dithiothreitol			Tyrosine formed (nmol)	
Time of addition ...	0	5	10	0	5	10	A	B
	−	−	−	−	−	−	10.2	6.2
	+	−	−	−	−	−	38.9	40.7
	−	+	−	−	−	−	25.0	20.5
	−	−	+	−	−	−	22.2	19.9
	−	−	−	+	−	−	27.3	21.6
	+	−	−	−	+	−	47.7	51.0
	+	−	−	−	−	+	51.0	47.2
	−	+	−	+	−	−	30.7	23.4
	−	−	+	+	−	−	23.8	24.4

Table 4. *Stability of dimethyltetrahydropterin in solution: effect of later addition of catalase and dithiothreitol*

Dimethyltetrahydropterin, 0.3 μmol, NADH, 0.8 μmol, and nicotinamide, 5 μmol in 0.6 ml of 0.2 M-sodium phosphate buffer, pH 6.8, was preincubated at 25°C (except where shown) for various times, then catalase and/or dithiothreitol was added. Rat liver supernatant (0.1 ml of the 1:28 homogenate), phenylalanine (2 μmol) and buffer to a volume of 1 ml were added and incubation continued for a further 30 min at 25°C.

	Addition at incubation stage		Tyrosine formed (nmol)				
Preincubation time (min) ...	Catalase (units)	Dithiothreitol (μmol)	0	15	30	45	45*
	0	0	26	20.5	13	0	18.5
	1830	0	55.5	35.0	13	0	36.0
	0	10	55.5	41.0	16.5	0	—
	1830	10	60.0	45.0	21.3	0	53.5

* Preincubation for 45 min was at 0°C.

Table 5. *Stability of dimethyltetrahydropterin in solution: effect of catalase, dithiothreitol and NADH*

Dimethyltetrahydropterin (0.3 μmol), was preincubated with nicotinamide (5 μmol) in 1 ml of 0.2 M-sodium phosphate buffer, pH 6.8, for 45 min at 25°C. Then phenylalanine (2 μmol) and rat liver supernatant (0.1 ml of the 1:28 homogenate) were added and incubation was continued for a further 30 min at 25°C. Catalase (1830 units), dithiothreitol (5 μmol) and NADH (0.8 μmol) were added at either the preincubation or the incubation stage as indicated. The control tubes were not preincubated.

Addition		Tyrosine formed (nmol)	
Preincubation	Incubation	Experimental	Control
Nil	NADH, catalase and dithiothreitol	0	55.5
Catalase	NADH and dithiothreitol	0	55.5
Dithiothreitol	NADH and catalase	55	55.5
NADH+liver supernatant	Catalase and dithiothreitol	29.5	34*
Liver supernatant	NADH, catalase and dithiothreitol	9	34*
NADH	Catalase and dithiothreitol	0	55.5

* The control tubes contained 0.1 ml of liver supernatant; this had a lower protein content and enzymic activity (60%) than in the other 4 sets of tubes.

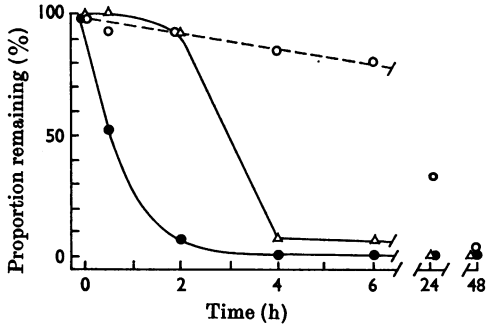

Fig. 4. Oxidation of dithiothreitol and inactivation of dimethyltetrahydropterin on incubation together aseptically after sterile filtration. ○, Percentage of dithiothreitol (initially 5mM) remaining after incubation for various times at 25°C in 0.2M-sodium phosphate buffer, pH6.8, with 10mM-phenylalanine, 5mM-nicotinamide and 0.8mM-NADH with shaking in air; ●, percentage of dithiothreitol (initially 5mM) remaining after incubation as above but with added 0.5mM-cofactor; △, percentage of cofactor (initially 0.5mM) remaining.

activation of dimethyltetrahydropterin by oxidation nor regenerate the active tetrahydropterin from the quinonoid dihydropterin. The mechanism by which NADH in the presence of liver supernatant protects the cofactor (but not phenylalanine hydroxylase) is presumably reduction by NADH of the quinonoid dihydropterin, catalysed by dihydropteridine reductase; it would seem that this enzyme is more resistant than phenylalanine hydroxylase to inactivation by the peroxide(s) formed from dimethyltetrahydropterin and oxygen.

We thank Dr Shirley Su Gillam for many helpful discussions. This investigation was supported by a grant from the Medical Research Council of Canada.

REFERENCES

Bublitz, C. (1969). *Biochim. biophys. Acta*, **191**, 249.
Ellman, J. L. (1959). *Archs Biochem. Biophys.* **82**, 70.
Jakubovič, A. (1971). *Biochim. biophys. Acta*, **237**, 469.
Kaufman, S. (1959). *J. biol. Chem.* **234**, 2677.
Kaufman, S. (1962). In *Oxygenases*, p. 129. Ed. by Hayaishi, O. New York: Academic Press Inc.
Kaufman, S. (1964). *J. biol. Chem.* **239**, 332.
Kaufman, S. (1970). *J. biol. Chem.* **245**, 4751.
Phillips, R. E. (1967). In *Manual of Fluorometric Procedures*. Palo Alto: G. K. Turner Associates Inc.
Viscontini, M., Leidner, H., Mattern, G. & Okada, T. (1966). *Helv. chim. Acta*, **49**, 1911.
Waalkes, T. P. & Udenfriend, S. (1957). *J. Lab. clin. Med.* **50**, 733.
Warburg, O. & Christian, W. (1941). *Biochem. Z.* **310**, 384.
Woolf, L. I., Jakubovič, A., Woolf, F. & Bory, P. (1970). *Biochem. J.* **119**, 895.
Zannoni, V. G. & Moraru, E. (1970). *FEBS Symp.* vol. 19: *Metabolic Regulation and Enzyme Action*, p. 347. Ed. by Sols, A. & Grisolia, S. London: Academic Press (Inc.) Ltd.