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The Hydroxylation of Tyrosine by an Enzyme from Third-Instar Larvae of the Blowfly *Calliphora erythrocephala*

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1. Two pro-(phenol oxidases) were distinguished when the blood of late-third-instar larvae of *Calliphora erythrocephala* was electrophoresed in polyacrylamide gels with Tris–glycine buffer, pH8.3. One pro-(phenol oxidase), after activation by an enzyme, readily catalyses the oxidation of both L-tyrosine and L-3,4-dihydroxyphenylalanine (L-dopa). The second enzyme catalyses the oxidation of L-dopa but not of L-tyrosine. 2. One of the pro-(phenol oxidases) was purified over 2000-fold from homogenates of whole larvae. This enzyme, after activation, catalyses the oxidation of both dopa and tyrosine. On electrophoresis in polyacrylamide gels with Tris–glycine buffer, pH8.3, it has the same mobility as the enzyme in the blood which catalyses the oxidation of both tyrosine and dopa. 3. The pro-(phenol oxidase)-activating enzyme was purified over 100-fold from homogenates of whole larvae. 4. The oxidation of L-tyrosine, in the presence of the activated purified phenol oxidase, reached a steady maximum rate after a lag period that was directly related to tyrosine concentration and inversely related to enzyme concentration. 5. The effect of the addition of electron donors on the lag period was studied. Dopa, dopamine (3,4-dihydroxyphenethylamine) and 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine are the most effective hydrogen donors. 3,4-Dihydroxybenzoic acid, the oxidation of which was not catalysed by the activated pro-(phenol oxidase), did not affect the lag period.

Tyrosine metabolism in insects has received much attention because of its special importance in the tanning of the cuticle at each moult. In the third-instar larvae of the blowfly *Calliphora erythrocephala* (Meig.), phenol oxidases (EC 1.14.18.1, *o*-diphenol–O_2 oxidoreductase) in the blood are responsible for most of the phenol oxidase activity (Sekeris & Mergenhagen, 1964). In *Calliphora*, as in other insects, the phenol oxidases of the blood are at this stage in the form of inactive pro-enzymes, which can be activated by an enzyme that can be obtained from extracts of the cuticle (Schweiger & Karlson, 1962; Ohnishi, 1954, 1958; Ohnishi *et al.*, 1970). Three pro-(phenol oxidases) have been distinguished in the blood of the fruitfly *Drosophila*. Two have almost identical electrophoretic mobilities when electrophoresed in polyacrylamide gels with 0.1M-sodium phosphate buffer, pH6.3, and both of them oxidize L-dopa (3,4-dihydroxyphenylalanine) but not tyrosine; they are dopa oxidases. The third, with a different electrophoretic mobility, oxidizes both dopa and tyrosine; it is a tyrosinase (Mitchell & Weber, 1965). These two classes of phenol oxidase, dopa oxidase and tyrosinase respectively, were also distinguished when the blood from larvae of three species of *Calliphora* was electrophoresed in gradient-pore polyacrylamide gels (Sin & Thompson, 1971).

The present paper describes the separation of pro-(phenol oxidases) of the blood of late-third-instar *Calliphora erythrocephala* larvae by electrophoresis in polyacrylamide gels with Tris–glycine buffer, pH8.3. It describes how a pro-(phenol oxidase), as well as the activating enzyme, can be purified from whole larvae without having recourse to separating the blood and the cuticle of the larvae. Some of the properties of the hydroxylation of tyrosine in the presence of the activated purified pro-(phenol oxidase) have been studied.

Experimental

Materials

Insects. Larvae of *Calliphora erythrocephala* were grown on ox spleen at 28°C, collected in sawdust at the late wandering stage, which precedes pupation, and stored at 4°C for up to 3 weeks, until the required quantity of larvae had been accumulated.

Chemicals. Bovine serum albumin and Dowex 50W (H^+ form; 4% cross-linked; 200–400 dry mesh) was obtained from Sigma (London) Chemical Co., Kingston-upon-Thames, Surrey, U.K. Dimethyltetrahydropterin (2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine) was obtained from Aldrich Chemical Co., Milwaukee, Wis. 53233, U.S.A.

Tetrahydrobiopterin [2-amino-4-hydroxy-6-(1,2-dihydroxypropyl)pteridine] was obtained from Roche Products Ltd., Welwyn Garden City, Herts., U.K. L-[3,5-^{3}H]Tyrosine was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Scintillation chemicals {PPO (2,5-diphenyloxazole) and POPOP [1,4-bis-(5-phenyloxazol-2-yl)benzene]} were obtained from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K. Sephadex G-25 (coarse grade) and Sephadex G-100 (medium grade) were obtained from Pharmacia (G.B.) Ltd., London W5 5SS, U.K. DEAE-celluloses were obtained from H. Reeve Angel and Co. Ltd., London EC4, U.K., and precycled immediately before use. All other chemicals were obtained from BDH Chemicals Ltd., Poole, Dorset, U.K. In the initial stages of enzyme purification, reagent-grade $(NH_4)_2SO_4$ was used for protein precipitation, but, after chromatography on DEAE-cellulose, AnalaR-grade $(NH_4)_2SO_4$ was used. All other reagents were AnalaR grade.

Buffers. Stock solutions of buffers were made by adjusting the pH of 0.5M-Tris with 11.6M-HCl and by adjusting the pH of 0.5M-Na_2HPO_4 with 0.5M-NaH_2PO_4. They were diluted with water to give buffers of the required concentrations.

Methods

Enzyme assays. All the enzyme assays were carried out at 25°C. Oxidation of tyrosine and dopa was assayed spectrophotometrically by measuring the rate of formation of dopachrome (2-carboxy-2,3-dihydroindole-5,6-quinone) by the increase of absorbance at 475nm during the first 2min of the reaction. Solutions of tyrosine and dopa were in 10mM-Tris–HCl buffer, pH7.0, and reactions were carried out with a total volume of 1ml in a 1cm-light-path cuvette. In calculating the enzyme unit, the molar extinction of dopachrome was taken as 3600 (Mason, 1948). The enzyme unit was taken as the amount of enzyme which will catalyse the oxidation of 1μmol of L-dopa/min at 25°C, and it was assumed that 1μmol of dopachrome is produced for every 2μmol of dopa oxidized (Evans & Raper, 1937; Fling *et al.*, 1963). In the presence of the activated purified pro-(phenol oxidase) DL-dopa was found to be oxidized 0.6 times as fast as L-dopa. The values obtained when DL-dopa was used as a substrate were corrected accordingly. When tyrosine was the substrate it was assumed that every mol of dopa formed was oxidized to dopachrome.

Hydroxylation of tyrosine was measured radiochemically by measuring the rate of formation of ^3H_2O from L-[3,5-^{3}H]tyrosine (Nagatsu *et al.*, 1964). The reaction mixture in every case contained the following, in a total volume of 1ml: 100μmol of sodium phosphate buffer, pH7.0 (by addition of 2M-sodium phosphate buffer, pH7.0), L-[3,5-^{3}H]tyrosine [specific radioactivity 1Ci/mmol; about 100000c.p.m. under the conditions for radioactivity counting given below (16ml of scintillation fluid and 2.5ml of water)], L-tyrosine (0.1μmol in 200μl), and enzyme (in 50μl). When dimethyltetrahydropterin was used as an electron donor, it was dissolved in 0.005M-HCl. The reaction mixture was shaken during incubation and the reaction stopped by the addition of 50μl of 10% (w/v) trichloroacetic acid. The mixture was then cooled in ice and, if necessary, centrifuged at low speed to remove precipitated protein. The contents of the tube, or the supernatant after centrifugation, were transferred to a column of 1ml of Dowex 50 (H^+ form), overlaid with 40mg of charcoal, in a Pasteur pipette plugged with glass wool. The first 0.5ml of eluate, which is less than the void volume of the column, was discarded. The total remaining eluate, including 2ml of water used to wash the column, was collected into 16ml of scintillation fluid, a mixture of Triton X-100 (scintillation grade) and toluene (1:2, v/v) containing 6g of PPO/litre and 0.1g of POPOP/litre. The mixture was shaken thoroughly and the radioactivity counted in a Beckman 120C liquid-scintillation counter. Blank values with boiled enzyme were about 1000c.p.m.

Before it was assayed, the pro-(phenol oxidase) was activated by incubating it at 20°C for 20min with an equal volume of the activating enzyme. The solution of activating enzyme, in 10mM-Tris–HCl buffer, pH7.0, with 10mM-$CaCl_2$, contained 430 *N*-α-benzoyl-L-arginine ethyl ester units/ml. The activating enzyme has tryptic activity and this was measured by following the hydrolysis of *N*-α-benzoyl-L-arginine ethyl ester spectrophotometrically. A portion (0.1ml) of a solution containing the activating enzyme was added to 2.5ml of buffer (0.2M-Tris–HCl–0.02M-$CaCl_2$, pH8.2) and 0.1ml of 0.01M-*N*-α-benzoyl-L-arginine ethyl ester, and the change in E_{253} was recorded. One unit of enzyme activity is defined as the amount of enzyme required to hydrolyse 1μmol of *N*-α-benzoyl-L-arginine ethyl ester/min at 25°C.

The activity of the activating enzyme was also assayed by determining the dopa oxidase activity of a mixture of 1 vol. of activator and 1 vol. of pro-(phenol oxidase) (about 7500 units/ml) after incubation for 5min at 20°C.

Gel electrophoresis. Polyacrylamide-disc-gel electrophoresis in the presence of sodium dodecyl sulphate was performed as described by Weber & Osborn (1969), except that the final concentration of acrylamide in the gels was 5% (w/v). The proteins used as standards of known molecular weight were: myosin, 200000; phosphorylase, 94000; bovine serum albumin, 68000; catalase, 60000; ovalbumin, 43000; haemoglobin, 15500; and lysozyme, 14300. Polyacrylamide-disc-gel electrophoresis with 0.1M-Tris–glycine buffer, pH8.3, was performed by the method of Davis (1964); the final concentration of

acrylamide was varied as indicated. Gels were stained for protein with Coomassie Brilliant Blue and scanned at 650nm by using a Gilford 240 spectrophotometer. Phenolase activity in the gels was detected by first incubating them at room temperature (about 20°C) in a solution of activating enzyme, containing 430 *N*-α-benzoyl-L-arginine ethyl ester units/ml of 10mM-Tris–HCl buffer, pH7.0, for 15min. They were then transferred to a solution of either 1mM-L-tyrosine or 25mM-DL-dopa in 10mM-Tris–glycine buffer, pH7.0, and incubated at room temperature for 20min. Ovalbumin, bovine serum albumin, aldolase, xanthine oxidase, phosphorylase and ferritin were used as molecular-weight standards for the determination of molecular weight from electrophoretic mobility by the method of Hendrick & Smith (1968).

For electrophoresis of larval blood, 100μl was collected in a calibrated glass capillary tube after pricking the anterior end of the larva with a pin. It was mixed with 100μl of 0.1M-Tris–glycine buffer, pH8.3, and 50μl of an aq. 50% (v/v) solution of glycerol, containing 1% Bromophenol Blue: 10μl of this mixture was electrophoresed on each gel.

Protein concentrations. Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin (containing less than 1.5% water, w/w) as a standard.

Results

Electrophoresis of pro-(phenol oxidases) of the blood

When the blood of *Calliphora* late third-instar larvae is electrophoresed in polyacrylamide gels with 0.1M-Tris–glycine buffer, pH8.3, two pro-(phenol oxidases) which catalyse the oxidation of dopa are separated (Fig. 1*a*). The band of activity with a lower mobility (Fig. 1*a*, band 1) is broader than the second band (Fig. 1*a*, band 2). When incubated in 1mM-tyrosine for up to 4h, only band 1 activity is found to catalyse the oxidation of tyrosine. The mobility of the pro-phenolase, isolated as described below, is identical with the mobility of band 1 when electrophoresed in gels with acrylamide concentrations of 9 and 4% (w/v) in 0.1M-Tris–glycine buffer, pH8.3. This was confirmed by electrophoresing the purified enzyme and blood on the same gel.

Purification of pro-(phenol oxidase)

As the pro-(phenol oxidase) is stable at room temperature, all procedures were performed at room temperature, except for column chromatography and homogenization, which were carried out at 4°C. All centrifugation was at 30000*g* for 30min.

Larvae were washed with several changes of tap water and homogenized in cold 10mM-Tris–HCl buffer, pH7.0, which contained 10mM-$CaCl_2$ and was 40% saturated with $(NH_4)_2SO_4$. $CaCl_2$ was included to preserve the activity of the activating enzyme. A 2.5-litre Waring blender was used for 1min at both medium and high speeds: 1 litre of buffered $(NH_4)_2SO_4$ solution was used for every 250g of larvae. The homogenate was filtered through cheesecloth and centrifuged. The precipitate was reserved for purification of the activating enzyme, which retains its activity for at least 3 months when stored at −20°C. Solid $(NH_4)_2SO_4$ was added to the supernatant with constant stirring to obtain 75% saturation. The precipitate formed was collected by filtration overnight, under vacuum, with Whatman no. 50 filter paper (diameter 50cm).

The filtered precipitate was suspended in 20mM-Tris–HCl buffer, pH7.0 (3 litres), and heated with constant stirring in a water bath at 60°C, until the temperature of the suspension reached 55°C, when it was transferred to an ice bath and cooled to room

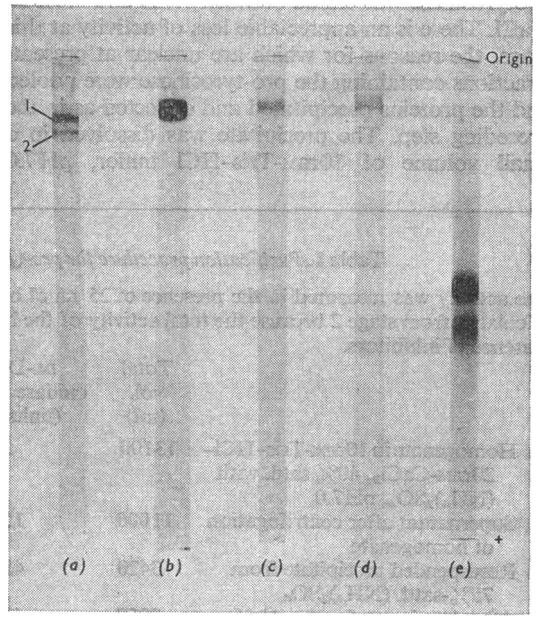

Fig. 1. *Polyacrylamide-gel electrophoresis of larval blood and of purified pro-(phenol oxidase)*

(*a*) Blood electrophoresed in a gel containing 7.5% (w/v) acrylamide with 0.1M-Tris–HCl buffer, pH8.3, and stained for phenol oxidase activity after incubation with activating enzyme. (*b*)–(*d*) Purified pro-(phenol oxidase) electrophoresed under the same conditions and stained for protein (*b*), for activity with L-tyrosine as substrate (*c*), and for activity with DL-dopa as substrate (*d*). (*e*) Purified pro-(phenol oxidase) electrophoresed in presence of 0.1% sodium dodecyl sulphate in a gel containing 5% (w/v) acrylamide and stained for protein.

temperature. The precipitate was then removed by centrifugation.

The supernatant (3050ml) was desalted on a column (9cm×140cm) of Sephadex G-25, equilibrated with 10mM-Tris–HCl buffer, pH8.0. The desalted proteins (8100ml) were chromatographed on a column (13cm×20cm) of DEAE-cellulose (Whatman DE11), equilibrated with 10mM-Tris–HCl buffer, pH8.0, and eluted with 4 litres of a linear gradient of 0–0.4M-NaCl in 10mM-Tris–HCl buffer, pH8.0. The pro-tyrosinase was eluted between 0.035 and 0.046M-NaCl.

The fractions containing the pro-enzyme were pooled, saturated with solid $(NH_4)_2SO_4$, and centrifuged. The precipitated proteins were dissolved in 10mM-Tris–HCl buffer, pH8.0 (145ml), and desalted on a column (9cm×140cm) of Sephadex G-25, equilibrated with the same buffer. The desalted proteins (440ml) were rechromatographed on a column (4.5cm×33cm) of DEAE-cellulose (Whatman DE52), which was eluted with 2 litres of a linear gradient of 0–0.1M-NaCl in 10mM-Tris–HCl buffer, pH8.0. The protyrosinase was eluted between 0.026 and 0.045M-NaCl. There is an appreciable loss of activity at this stage, the reasons for which are unclear at present. Fractions containing the pro-tyrosinase were pooled and the proteins precipitated and collected as in the preceding step. The precipitate was dissolved in a small volume of 10mM-Tris–HCl buffer, pH7.0 (about 10ml), and chromatographed on a column (2.5cm×80cm) of Sephadex G-100, both equilibrated and eluted with 10-mM-Tris–HCl buffer, pH7.0.

Further chromatography on a column (2.2cm×3cm) of hydroxyapatite, eluted with 250ml of a 0–0.5M gradient of potassium phosphate buffer, pH7.0, may be necessary: but if fractions containing specific activities lower than 200 DL-dopa oxidase units/ml are rejected in the two preceding steps, chromatography on hydroxyapatite gives no further purification. The pro-tyrosinase was eluted from hydroxyapatite between 0.05 and 0.16M-potassium phosphate.

Table 1 summarizes the results of a typical purification.

Purification of the activating enzyme

$CaCl_2$ (10mM) was added to all solutions in order to maintain the activity of the enzyme. The conditions of temperature and centrifugation were the same as for the purification of the pro-(phenol oxidase). The precipitate from larvae which had been homogenized in buffered 40%-satd. $(NH_4)_2SO_4$, as described in the first step of the purification of the pro-(phenol oxidase), was suspended in 10-mM-Tris–HCl buffer, pH8.0 (300ml), and centrifuged. The supernatant was dialysed against several changes of 10 litres of 10mM-Tris–HCl buffer, pH8.0, until its conductivity

Table 1. *Purification procedure for pro-(phenol oxidase) from 2.45kg of Calliphora larvae*

The activity was measured in the presence of 25 μmol of DL-dopa as described in the Experimental section. The yield is calculated from stage 2 because the total activity of the homogenate is much lower than at stage 2, possibly owing to the presence of inhibitors.

	Total vol. (ml)	DL-Dopa oxidase activity (units/ml)	Protein (mg/ml)	10^{-2}×Total activity (units)	Specific activity (units/mg of protein)	Yield (%)
(1) Homogenate in 10mM-Tris–HCl–20mM-$CaCl_2$, 40% satd. with $(NH_4)_2SO_4$, pH7.0	13100	34	91.5	445	0.37	—
(2) Supernatant after centrifugation of homogenate	11000	157	79.8	1727	1.98	100
(3) Resuspended precipitate from 75%-satd. $(NH_4)_2SO_4$	3420	453	255.7	1549	1.77	89
(4) After heating and removal of precipitate by centrifugation	3050	490	164.0	1495	2.98	90
(5) Eluate after desalting on Sephadex G-25	8100	150	59.3	1215	2.52	76
(6) Eluate from DE11 DEAE-cellulose column chromatography	760	1685	24.6	1281	68.5	74
(7) Eluate after desalting on Sephadex G-25	440	2659	49.1	1170	54.2	69
(8) Eluate from DE52 DEAE-cellulose column chromatography	220	1536	3.2	338	480	19
(9) Eluate from Sephadex G-100 chromatography	36	7490	1.81	270	4138	10

Table 2. *Purification procedure for the pro-(phenol oxidase)-activating enzyme*

The preparation was from 145g of the precipitate obtained after centrifugation of a homogenate of *Calliphora* larvae in buffered 40%-satd. $(NH_4)_2SO_4$ (Table 1, step 2). Activity cannot be measured at stage 1 owing to the high ionic strength of the supernatant before dialysis.

	Total vol. (ml)	Activity (dopa oxidase units/ml)	Protein (mg/ml)	$10^{-3}\times$Total activity (units)	Specific activity (units/mg of protein)	Yield (%)
(1) Supernatant after centrifugation of 40%-satd. $(NH_4)_2SO_4$ precipitate	310	—	61.2	—	—	—
(2) Supernatant after dialysis	390	827	25.0	322	33	100
(3) Supernatant after centrifugation of dialysed supernatant from Stage 2	380	613	11.8	233	52	72
(4) Eluate from DE11 DEAE-cellulose column chromatography	600	347	0.74	208	469	64
(5) Eluate from DE52 DEAE-cellulose column chromatography	173	433	0.13	75	3330	23

was below $1\,\Omega^{-1}$. It was then centrifuged. The supernatant (380ml) was applied to a column (4.5cm× 60cm) of DEAE-cellulose (Whatman DE11), equilibrated with 10mM-Tris–HCl buffer, pH8.0, and eluted with a 3-litre linear gradient of 0–0.5M-NaCl, in the same buffer. The activating enzyme, which was eluted between 0.02 and 0.07M-NaCl, had no *o*-diphenol oxidase activity. The fractions containing the activating enzyme were pooled, saturated with solid $(NH_4)_2SO_4$ and centrifuged. The precipitate was dissolved in 10mM-Tris–HCl buffer, pH8.0 (140ml), and desalted on a column (7.5cm×53cm) of Sephadex G-25. The desalted proteins (510ml) were rechromatographed on a column (2.4cm×66cm) of DEAE-cellulose (Whatman DE52), equilibrated with 10mM-Tris–HCl buffer, pH8.0, and eluted with 1.5 litres of a linear gradient of 0–0.2M-NaCl in the same buffer. The activating enzyme was eluted between 0.02 and 0.05M-NaCl. Table 2 summarizes the results of a typical purification.

Gel electrophoresis of the purified pro-tyrosinase

Polyacrylamide-gel electrophoresis of the purified pro-tyrosinase, in the presence of sodium dodecyl sulphate, showed two bands when stained for protein, which were by densitometry in the ratio of 1.4:1, and had mol.wts. corresponding to 100000 and 90000 respectively (Fig. 1*e*). Polyacrylamide-gel electrophoresis of the purified pro-(phenol oxidase) with 0.1M-Tris–glycine buffer, pH8.3, at acrylamide concentrations of 9, 8, 7, 6, 5 and 4% (w/v), all gave a single band when incubated, after activation, in either dopa or tyrosine (Figs. 1*c* and 1*d*), and a single band when electrophoresed in 7% acrylamide gels and stained for protein (Fig. 1*b*).

When proteins are electrophoresed in gels of different acrylamide concentrations there is a linear relationship between the log of protein mobility and the acrylamide concentration of the gel. The slope of the plot of the log of mobility against acrylamide concentration is directly related to molecular weight. The molecular weight of a protein can therefore be determined from its mobility on electrophoresis in gels of different acrylamide concentrations, once the relationship between slope and molecular weight has been determined with proteins of known molecular weight (Hendrick & Smith, 1968). By this method the molecular weight of the pro-(phenol oxidase) was 100000.

Oxidation of tyrosine and dopa

The activated purified pro-(phenol oxidase) readily catalyses the oxidation of both tyrosine and dopa. The oxidation of tyrosine was inhibited by substrate concentrations above 1mM (Fig. 1). By measuring the formation of dopachrome the K_m value for tyrosine was found to be 0.41 ± 0.04mM and the K_m value for the oxidation of dopa was 3.3 ± 2.0mM.

Effect of tyrosine concentration and enzyme concentration on the lag period in the hydroxylation of tyrosine

In the absence of added electron donors, hydroxylation of tyrosine proceeds at a steady rate after a lag period. This is estimated by extrapolation of the steady-state portion of the curve of activity against time to the abscissa, the lag period being taken as the time at which the extrapolated line cuts the abscissa. The lag period lengthened with increasing concentration of tyrosine (Fig. 3). The inverse of the lag period

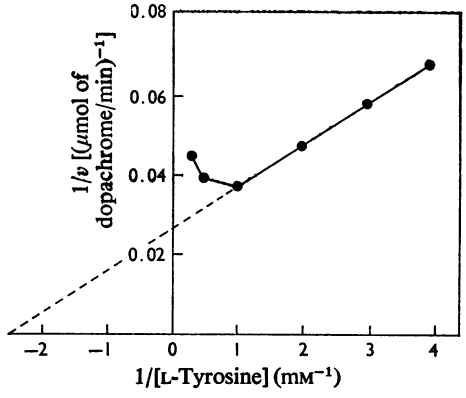

Fig. 2. *Double-reciprocal plot of the steady-state velocity of dopachrome formation against* L-*tyrosine concentration*

Activated pro-(phenol oxidase) (20 units) was incubated with various concentrations of tyrosine under the conditions described in the Experimental section. The line shown is calculated by using the statistical treatment of Wilkinson (1961).

varied linearly with enzyme concentration for the concentrations used (Fig. 4).

Effect of electron donors on the hydroxylation of tyrosine

The addition of many electron donors decreases the length of the lag period. By using the method of Pomerantz & Warner (1967) to calculate the amount of electron donor required to halve the lag period observed during the spectrophotometric determination of tyrosine hydroxylation, the amount of L-dopa required was found to be 3×10^{-6}M and the amount of dimethyltetrahydropterin required was 16×10^{-6}M (Fig. 5). Under the same conditions the lag period was almost eliminated in the presence of 5μM-dopamine. In the case of dimethyltetrahydropterin, the linear relationship between the inverse of the lag period and the amount of added electron donor only held for low concentrations of added electron donor. At a concentration of 50μM-dimethyltetrahydropterin the lag period (2.6min) was longer than that calculated (1.1min) by extrapolation of the linear plot of the inverse of the lag periods against low concentrations of electron donor. When the hydroxylation of tyrosine in the presence of various amounts of dimethyltetrahydropterin was followed radiochemically there was similarly a decrease in the lag period at low concentrations of dimethyltetrahydropterin. The progress curves became complex at high concentrations of dimethyltetrahydropterin. At all concentrations there was an increase in the rate of hydroxylation of tyrosine which was dependent on the amount of

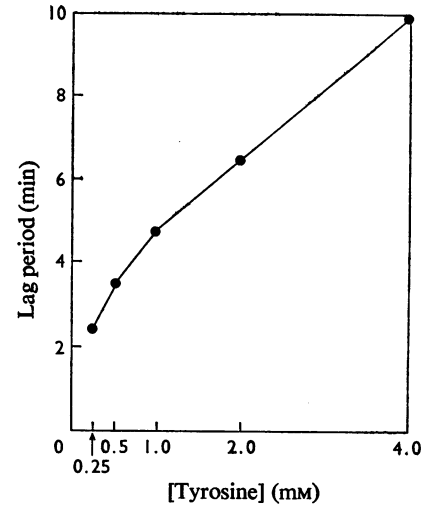

Fig. 3. *Effect of tyrosine concentration on the lag period of tyrosine hydroxylation*

Activated pro-(phenol oxidase) (20 units) was incubated with various concentrations of L-tyrosine as described in the Experimental section.

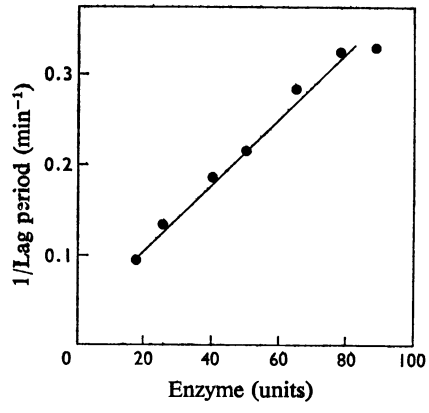

Fig. 4. *Effect of enzyme concentration on the lag period observed in tyrosine hydroxylation*

L-Tyrosine (1μmol) was incubated with various concentrations of activated pro-(phenol oxidase) as described in the Experimental section.

added dimethyltetrahydropterin (Fig. 6). The relative effectiveness of different electron donors for the hydroxylation of tyrosine was compared by measuring the amount of dopa produced in 2.0min in the presence of 25μmol of electron donor (Table 3, column *a*). In another experiment the amount of electron donor required to produce the half-maximal increase

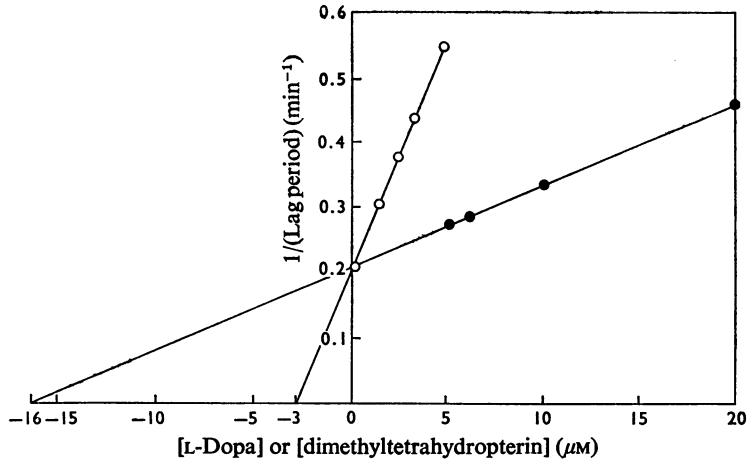

Fig. 5. *Relationship between the reciprocal of the lag period for the oxidation of tyrosine and the concentration of added dopa* (○) *and dimethyltetrahydropterin* (●)

Activated pro-(phenol oxidase) (18.4 units) was incubated with 1 μmol of L-tyrosine and various concentrations of L-dopa and dimethyltetrahydropterin under the conditions described in the Experimental section, and the lag period was measured.

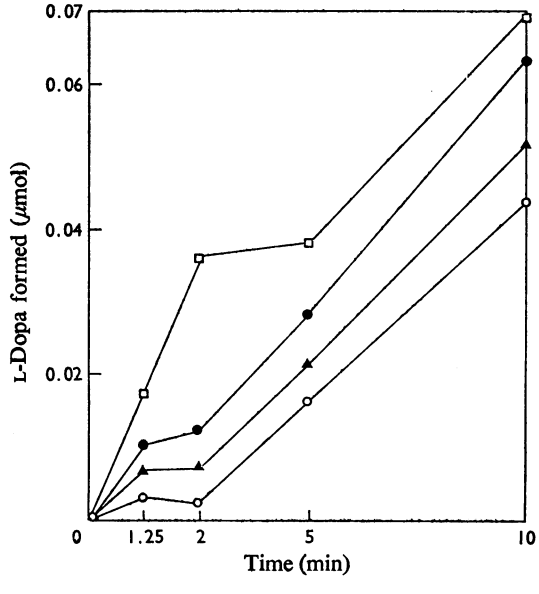

Fig. 6. *Rate of formation of* L-*dopa from* L-*tyrosine in the presence of various concentrations of dimethyltetrahydropterin*

Activated pro-(phenol oxidase) (20 units) was incubated with 1 μmol of L-tyrosine and various concentrations of dimethyltetrahydropterin under the conditions described in the Experimental section. The formation of L-dopa was determined radiochemically. Concentrations of dimethyltetrahydropterin: ○, 0; ▲, 5 μM; ●, 10 μM; □, 50 μM.

in dopa formed after 2.5 min incubation was calculated from the statistical best fit of the assay results to the Michaelis–Menten equation (Table 3, column *b*). By both these methods dopa, dopamine and dimethyltetrahydropterin were found to be the most effective electron donors for tyrosine hydroxylation. The values calculated must only be regarded as an approximate indication of the relative effectiveness of the various electron donors because of the complexity of the progress curves. No stimulation of tyrosine hydroxylation was observed in the presence of added 3,4-dihydroxybenzoic acid, which is not oxidized by this enzyme.

Discussion

Two earlier accounts of the properties of phenol oxidases from blowflies differ strikingly with regard to the substrate specificities of the enzymes. Karlson & Liebau (1961) extracted an enzyme from acetone-dried powders of whole larvae which oxidized dopa readily but hardly oxidized tyrosine. Hackman & Goldberg (1967) found that the larval blood of *Lucilia cuprina* oxidized both tyrosine and dopa. There are many other reports of phenolases extracted from whole insect larvae which show strong monophenolase activity (Raper, 1926; Bodine *et al.*, 1937; Ohnishi, 1953; Ito, 1953; Horowitz & Fling, 1955). The results in the present paper show that in *Calliphora*, as in *Drosophila* (Mitchell & Weber, 1965), two pro-(phenol oxidases) can be distin-

Table 3. *Electron donors for tyrosine hydroxylation*

(*a*) Electron donor (25 nmol) was incubated for 2 min with 1 μmol of tyrosine in the presence of 42 units of activated phenol oxidase under the conditions described in the Experimental section, and the amount of dopa produced measured. (*b*) Various concentrations of electron donor were incubated for 2.5 min with 20 units of activated pro-(phenol oxidase) under the same conditions and the amount required to produce a half-maximal increase in dopa was calculated.

Electron donor	(*a*) Dopa formed (μmol)	(*b*) Concn. of electron donor required to produce half-maximal increase in dopa (μM)
Dopamine	0.136	1.5±0.5
L-Dopa	0.098	5± 1
Dimethyltetrahydropterin	0.084	26± 5
Tetrahydrobiopterin	0.061	60± 2
Ascorbate	0.040	86±11
NADH	0.038	1475±34
NADPH	0.028	1306±42
None	0.020	—

guished by electrophoresis in polyacrylamide gels; one is a diphenol oxidase, the other a tyrosinase.

The method developed for the preliminary purification of the pro-tyrosinase, as well as the activating enzyme, depends on using conditions at the beginning of the preparation which inhibit the activation of the pro-(phenol oxidase) by the activating enzyme. This was achieved in the first stage of the purification by homogenizing in buffer, 40% saturated with $(NH_4)_2SO_4$, which, because of its high ionic strength, inhibits the activating enzyme. Further, the activating enzyme is precipitated at this concentration of $(NH_4)_2SO_4$, whereas the pro-(phenol oxidase) is not. Because the preparation uses whole larvae instead of blood, large amounts of both enzymes can be obtained. This preparation is therefore particularly useful for small insects, such as flies. Only for the comparatively large larvae of the silkworm *Bombyx mori* have the pro-phenolase from the blood (Ashida, 1971) and the activating enzyme (Dohke, 1973) been well characterized. Attempts to isolate a phenolase from blowflies have not so far been fully satisfactory (Karlson & Liebau, 1961; Munn & Bufton, 1973; Hughes & Price, 1974).

Electrophoresis in sodium dodecyl sulphate of the phenolase, prepared as described here, indicates that at the final stage of purification there are still two proteins present, one or both of which could be the phenolase. Only a single active band was obtained when the purified phenolase was electrophoresed in Tris–glycine buffer, pH 8.3, on gels containing a wide variety of acrylamide concentrations. This suggests that only one of the two proteins is a phenolase. Further purification is necessary for their separation.

The isolation of the pro-phenolase described in this paper enables one to determine whether or not the pro-tyrosinase and pro-(dopa oxidase) of the blood that can be separated by electrophoresis are interconvertible. Sin & Thompson (1971) found that pro-tyrosinases and pro-(dopa oxidases) were clearly separated when the blood of larvae of three different species of *Calliphora*, including *C. vicina* (= *erythrocephala*), was electrophoresed on gradient-pore polyacrylamide gels in 0.1 M-Tris–0.04 M-boric acid–0.25 mM-EDTA, pH 8.8. But they further showed, in *C. stigia*, that when the pro-(diphenol oxidase) was eluted from the gel and re-electrophoresed under identical conditions, it gave rise to a band with the mobility and substrate specificity of the pro-tyrosinase in addition to the original band. Conversely, the pro-tyrosinase produced a pro-(dopa oxidase) on re-electrophoresis. The demonstration that the pro-(phenol oxidase) purified as described in the present paper has an electrophoretic mobility identical with that of one of the two bands obtained when larval blood was electrophoresed in polyacrylamide gels with 0.1 M-Tris–glycine buffer, pH 8.3, indicates that under these conditions the purified pro-(phenol oxidase) and the pro-(dopa oxidase) are distinct enzymes. This conclusion is supported by the finding that the purified pro-(phenol oxidase) gives a single band when electrophoresed in a wide range of polyacrylamide concentrations.

The effect of electron donors on the hydroxylation of tyrosine in the presence of phenolases varies according to the source of the phenolase. With a phenolase from an insect, the European Cornborer, *Ostrinia nubilalis*, dimethyltetrahydropterin did not affect hydroxylation (Retnakaran, 1969). In the presence of tyrosinase from hamster melanoma 2.4 mM-dimethyltetrahydropterin was required to eliminate the lag period, whereas the amount of dopa required was 40 μM (Pomerantz, 1966). On the other hand, dimethyltetrahydropterin proved to be the most effective electron donor for the hydroxylation of tyrosine in the presence of a phenolase from spinach

leaves (Vaughan & Butt, 1969). The results presented above show that dimethyltetrahydropterin, as well as dopa and dopamine, are effective electron donors for the hydroxylation of tyrosine in the presence of the phenolase isolated from fly larvae. Therefore if this enzyme is involved in the hydroxylation of tyrosine in insect blood, a pteridine could act as an electron donor. But at the same time it must be noted that the results also show that an electron donor other than the product of the reaction, dopa, may not be necessary, since the lag period is short at low tyrosine and high enzyme concentrations.

Although it is widely recognized that phenolases are an important component of insect blood there is no general agreement about their function. The enzyme isolated in the present study might be involved in one possible function, namely the conversion of tyrosine into diphenols that are used for cross-linking the cuticle at the moult. The demonstration that there are at least two phenolases with clearly distinct substrate specificities increases the possibility that the different phenolases of insect blood have clearly distinct roles yet to be determined.

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