Peroxidatic Oxidation of Tyrosine to Melanin in Supernatant of Crude Mouse Melanoma Homogenates

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Peroxide-dependent enzymic oxidation of tyrosine to dopachrome and melanin was demonstrated in cell-free melanoma homogenates. Histochemical methods for distinguishing peroxidase activity from aerobic dopa (3,4-dihydroxyphenylalanine) oxidase activity are not reliable with cell-free preparations. Therefore the presence of peroxidase activity in such preparations precludes assay of cresolase activity of mammalian 'tyrosinase'.

Because of the assumption that an aerobic tyrosinase is the only mammalian enzyme capable of oxidizing tyrosine to melanin (Duchon *et al.*, 1968), oxidation of tyrosine to melanin in crude or partially purified preparations of melanocyte-bearing tissue has been attributed to this enzyme without controls for the role of other oxidases (Chen & Chavin, 1969) or with controls which our experiments have shown to be ambiguous (Hearing, 1973) (see also under 'Discussion').

Experiments in our laboratory (Patel *et al.*, 1971) have shown that isolated plant and mammalian peroxidases can oxidize tyrosine (with cofactor) to melanin. Peroxidase-dependent oxidation of tyrosine to melanin was demonstrated in normal and neoplastic melanocytes (Okun, 1967; Okun *et al.*, 1970*a,b,c*, 1971, 1972, 1973*a,b*). Conversely, histochemical (Okun *et al.*, 1970*a,b,c*, 1972, 1973*b*) and biochemical (Patel *et al.*, 1973) experiments demonstrated that aerobic dopa (3,4-dihydroxyphenylalanine) oxidase in mammalian melanocytes, currently assumed to be tyrosinase (Burnett, 1971), is, as Bloch (1917) proposed, an aerobic dopa oxidase with no ability to oxidize tyrosine.

The present paper is concerned with the enzymic oxidation of tyrosine to melanin in crude mouse melanoma homogenates.

Experimental

1. *Preparation of crude supernatant.* This was carried out as described previously (Patel *et al.*, 1973) with additional centrifugation at 100000 g for 2.5h.

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2. *Spectrophotometric assay of oxidation of tyrosine to melanin.* Oxidation of L-tyrosine to dopachrome (2-carboxy-2,3-dihydroindole-5,6-quinone) was measured at 475nm with a Beckman DB-G recording spectrophotometer.

Reaction mixture A consisted of 0.2mg (11 μ mol) of L-tyrosine dissolved in 0.4ml of 0.1M-sodium phosphate buffer, pH 6.8, 0.4ml of crude supernatant and 0.41 ml of the above buffer.

Reaction mixture B differed from A by the addition of 0.011 ml (10 μ mol) of 3% (v/v) H₂O₂ and a decrease of the buffer to 0.4ml.

Reaction mixture C differed from A by the substitution of supernatant boiled for 30min.

Reaction mixture D was prepared as described below. A mixture of supernatant and buffer (1:1, v/v) containing 11mM-sodium diethyldithiocarbamate, was dialysed 4 times against 1 litre of water (30min each time). This mixture was then substituted for buffer and homogenate in reaction mixture B.

Residual sodium diethyldithiocarbamate in the dialysis residue was qualitatively determined by adding 1.0ml of 1% copper sulphate. (The reaction product of copper sulphate and sodium diethyldithiocarbamate is a yellow compound.) Copper sulphate was also treated with reaction mixture B as a control.

Reaction mixture E differed from A by the addition of 0.1ml of catalase (C-100; Sigma Chemical Co., St. Louis, Mo., U.S.A.; 2mg/ml) and the buffer volume was decreased to 0.31ml.

3. *Qualitative determination of peroxidase activity.* Peroxidase activity was determined in the supernatant by using the benzidine reaction of De Robertis & Grasso (Pearse, 1960).

4. *Polyacrylamide-gel electrophoresis.* This was carried out by the method of Davis (1964). The

following reactions were carried out on gels: (a) Coomassie Blue stain; (b) dopa oxidase reaction: gels were incubated in DL-dopa (100mg/100ml) in 0.1M-sodium phosphate buffer, pH 6.8, for 30–60min; (c) benzidine reaction of De Robertis & Grasso (Pearse, 1960); (d) bubble reaction for catalase: gels were incubated in 10mM-H₂O₂ and catalase was localized by a ring of bubbles.

Results

1. *Spectrophotometric assay of oxidation of tyrosine to melanin.* Without exogenous H₂O₂, an absorption curve was obtained at 475 nm (Fig. 1, curve a) after a lag period varying from 20 to 45 min. In a typical experiment, an initial peak ($E = 0.235$) was followed by a decrease and then a gradual rise. With exogenous H₂O₂ (Fig. 1, curve b), a curve of similar configuration was obtained, which had a much higher initial peak ($E = 1.1$ in a typical experiment) and a smaller lag period.

Fig. 1. Oxidation of L-tyrosine by crude mouse melanoma supernatant

The curves represent changes in E_{475} by the action of crude mouse melanoma supernatant on L-tyrosine. Curve (a), without added H₂O₂; curve (b), with added H₂O₂. The wavy terminal portions of curves are produced by suspension of insoluble melanin. For further details see the text.

Ultimately, suspensions of insoluble melanin formed in reactions with and without H₂O₂. With exogenous H₂O₂, a greater amount of insoluble melanin formed than without exogenous H₂O₂; this was visually evident.

No dopachrome or melanin formation was noted when boiled supernatant was used.

With reaction mixture D (supernatant preincubated with sodium diethyldithiocarbamate, followed by dialysis), the dopachrome absorption curve reached a maximum E of 0.29 in 45 min. However, the dialysis residue showed a yellow compound after treatment with 1% copper sulphate, indicating residual sodium diethyldithiocarbamate. No yellow compound formed when copper sulphate was added to reaction mixture B. With reaction mixture E (catalase included) there was no suppression of formation of dopachrome from tyrosine.

2. *Qualitative determination of peroxidase activity.* The supernatant showed a strongly positive benzidine reaction for peroxidase (deep-blue colour).

3. *Polyacrylamide-gel electrophoresis.* Four dopa-positive enzyme bands were present (Table 1). One was catalase, represented by a ring of bubbles in the presence of high concentrations of H₂O₂ (this band also produced a variably positive benzidine reaction); one was peroxidase, represented by a strongly positive benzidine reaction (a second, very faint, peroxidase band was also present); two were aerobic dopa oxidase ('tyrosinase'), represented by benzidine-negativity and absence of bubbles with H₂O₂.

Discussion

The similarity in the configuration of the dopachrome curves with and without added H₂O₂ suggests a peroxidatic mechanism for the rate-limiting first step of dopachrome formation (hydroxylation of tyrosine). Subsequent oxidation of dopa was due to the composite effect of peroxidase, aerobic dopa oxidase and catalase.

H₂O₂ is formed in the crude homogenate by enzymic oxidation (Knox, 1954) and by auto-oxidation of dopa (Gillette *et al.*, 1954) and other melanin intermediates (Swan & Wright, 1954).

Table 1. Correlated reactions of dopa-positive enzyme bands, demonstrated by polyacrylamide-gel electrophoresis, in crude melanoma supernatant

For details see the text. R_x , Ratio of the migration of enzyme band to the entire migration of Bromophenol Blue tracking dye.

Band	R_x value	Coomassie Blue stain	Dopa reaction	Benzidine reaction	O ₂ bubble test	Designation
A	0.15	+	+	±	+	Catalase
B	0.28	+	+	+	—	Peroxidase
b	0.36	+	+	+	—	Peroxidase
C	0.43	+	+	—	—	Aerobic dopa oxidase
D	0.55	+	+	—	—	Aerobic dopa oxidase

The transient decrease in dopachrome concentration after the initial peak may be related to accelerated breakdown of dopachrome by the initial burst of H_2O_2 formed by melanogenesis, since aerobic dopa oxidase ('tyrosinase') generates H_2O_2 without consuming it.

Sodium diethyldithiocarbamate in the incubation medium will non-selectively suppress peroxidase (Okun *et al.*, 1970a,b,c; Randall, 1946) activity as well as aerobic dopa oxidase ('tyrosinase') activity. In histochemical experiments, preincubation with sodium diethyldithiocarbamate followed by the thorough removal of it before exposure of sections to substrate, however, allows this thiol compound to selectively suppress aerobic dopa oxidase.

Assumptions that dialysis has removed all of the sodium diethyldithiocarbamate in experiments *in vitro* (Hearing, 1973) can lead to unwarranted conclusions; as noted, we found significant residual sodium diethyldithiocarbamate after using the identical dialysis procedures described by Hearing (1973).

Similarly, the effectiveness of catalase as an inhibitor of peroxidase in crude systems *in vitro* differs markedly from its effectiveness in histochemical systems. In histochemical systems, catalase in the incubation medium is present in overwhelmingly greater amounts than peroxidase in tissue sections, and possible peroxidase activity of catalase in the incubation medium would compete with, rather than add to, peroxidase activity in tissue sections. Conversely, as Knox (1954) has shown, considerable peroxidase activity can take place *in vitro* in the presence of catalase. As demonstrated in our experiments, considerable peroxidase activity was present in the presence of preformed catalase. Use of exogenous catalase in this system as an inhibitor of peroxidase (Hearing, 1973) is therefore ambiguous, particularly since catalase can have peroxidatic activity (Fahimi, 1969).

Attempts to differentiate peroxidase activity from 'tyrosinase' activity *in vitro* (Hearing, 1973) by using the histochemical inhibitor systems cited are therefore inconclusive, particularly when coupled with a radioassay system for melanin formation in which non-specific binding of labelled tyrosine to metal sites is not accounted for. It is known that tyrosine can bind metal sites without being oxidized (Okun *et al.*, 1970b; Shima, 1962).

Hearing (1973) concluded that increase in melanogenic activity with exogenous H_2O_2 could be non-specific (and not an indicator of peroxidase-dependency) since he noted a 10% increase in melanogenic activity of plant peroxidase with exogenous H_2O_2 . We agree that an increase of this order of magnitude could be non-specific; however, this does not apply

to our experiments since we observed an approx. 500% increase in melanogenic activity with added H_2O_2 in histochemical (Okun *et al.*, 1970b) and biochemical (see above) systems.

The present study indicates that, unless crude preparations of melanocyte-bearing tissue are free of peroxidase activity, oxidation of tyrosine to melanin cannot be attributed to aerobic dopa oxidase ('tyrosinase'). Since in our crude preparation oxidation of tyrosine to melanin was greatly increased by exogenous H_2O_2 , and since isolated mammalian 'tyrosinase' cannot oxidize tyrosine and isolated peroxidases can, it is likely that cresolase activity previously attributed to mammalian 'tyrosinase' in crude preparations was due to peroxidase.

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