A Study of the Supposed Hydroxylation of Tyrosine Catalysed by Peroxidase

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The claim that peroxidase (rather than tyrosinase) is the enzyme responsible for the conversion of tyrosine into dopa (3,4-dihydroxyphenylalanine) in melanogenesis was investigated. The spectral changes that occurred during the action of horseradish peroxidase in the presence of H_2O_2 on dopa, tyrosine and mixtures of dopa with tyrosine or other phenolic compounds were studied. The effect of ascorbic acid or dihydroxyfumaric acid on some of these changes was also investigated. No evidence was found that tyrosine was hydroxylated by peroxidase in the presence of H_2O_2 and dopa as cofactor, although tyrosine or other phenolic compounds increased the rate of oxidation of dopa to dopachrome (indoline-5,6-quinone-2-carboxylic acid). Peroxidase was, however, effective in oxidizing tyrosine to dopa in the presence of dihydroxyfumaric acid and oxygen.

According to the generally held views on the biogenesis of eumelanins, based on Raper's classical work, tyrosine is first hydroxylated to yield dopa (3,4-dihydroxyphenylalanine), which is then oxidized to dopaquinone, both steps being effected by the enzyme tyrosinase (EC 1.14.18.1) in the presence of molecular oxygen (Raper, 1928; Swan, 1974). Subsequent steps, which include the formation of dopachrome (indoline-5,6-quinone-2-carboxylic acid) and 5,6-dihydroxyindole, can be accomplished non-enzymically. However, from histochemical experiments Okun *et al.* (1970, 1972) concluded that peroxidase-dependent oxidation of tyrosine (in the presence of dopa as cofactor) or dopa to melanin occurs in mast cells, eosinophils, melanocytes and neurons. Moreover, from biochemical studies using isolated myeloperoxidase or horseradish peroxidase (EC 1.11.1.7) the same groups (Patel *et al.*, 1971, 1974) concluded that these two enzymes are able to convert tyrosine or dopa into melanin and that the first step involved the hydroxylation of tyrosine to dopa, catalysed by peroxidase in the presence of dopa as cofactor. They therefore proposed peroxidase rather than (or possibly in addition to) tyrosinase as the enzyme responsible for melanogenesis.

This theory is attractive because of the possibility that the H_2O_2 formed in the oxidation of dopa to dopaquinone might act as substrate for the peroxidase and because, in spite of much research, the mechanism of the hydroxylation of tyrosine to dopa by tyrosinase is not understood (Kertesz & Zito, 1962). Patel *et al.* (1973) failed to observe significant oxidation of tyrosine to melanin by mouse tyrosinase. However, in the course of attempted purification of

tyrosinase, other workers have observed that the enzyme developed an increasing lag period, or that the ratio of cresolase to catecholase activity diminished. One suggested explanation is the occurrence of an inactive form of tyrosinase, which requires activation (McGuire, 1970). Karkhanis & Frieden (1961) have actually isolated an inhibitor, and Cooper (1973) has demonstrated a substrate-binding inhibitor. Hearing & Ekel (1975) confirmed that mouse tyrosinase is capable of using tyrosine as substrate.

In view of the fundamental importance of the enzyme in melanogenesis we attempted to confirm the biochemical findings of Patel *et al.* (1971, 1974).

Experimental

Sigma type II horseradish peroxidase [RZ (Maehly, 1955) approx. 1.4] and dihydroxyfumaric acid were obtained from Sigma (London) Chemical Co Ltd., Kingston-upon-Thames, Surrey, U.K. L-Tyrosine and dopa (3,4-dihydroxy-DL-phenylalanine) (both chromatographically homogeneous) were obtained from B.D.H. Chemicals Ltd., Poole, Dorset, U.K. The hydrogen peroxide solution was approx. 0.1M. Di-*p*-cresol was synthesized by the method of Westerfeld & Lowe (1942). The following buffers, all of pH 5.6, were used: 0.2M- and 0.06M-acetic acid/sodium acetate; 0.06M-Sørensen's phosphate buffer (Washburn, 1926).

The buffers used in making up the various solutions are further specified in the Results and Discussion section. Soln. A contained 47mg of L-tyrosine in 100ml of buffer; Soln. B contained 12.2mg of

($\pm$)-dopa in 25ml of buffer; Soln. C contained 24.5mg of ($\pm$)-dopa in 25ml of buffer.

(a) When the absorption spectrum of the reaction mixture containing dopa, but in the absence of tyrosine, was to be measured, Soln. B (0.5ml) was mixed with buffer (10ml). When tyrosine was present, Soln. A (10ml) was mixed with Soln. B (0.5ml). The appropriate amino acid solution (3ml), together with H_2O_2 solution (0.25ml), was placed in the sample cell of the spectrophotometer. A solution of 1 mg of peroxidase in 0.25ml of water was added, and the absorption spectrum of the solution was scanned repeatedly at 2min intervals from 225 to 700nm. The reference cell contained 3ml of the appropriate amino acid solution with 0.5ml of water. In some experiments ascorbic acid (12.3 and 4.9mg) was added to Solns. A and B respectively.

(b) The formation of dopachrome was followed as described by Patel *et al.* (1974).

(c) In an additional set of experiments, in which the formation of dopachrome was followed, the spectrometer was set at 475nm and the enzyme solution contained 32.4mg of peroxidase in 4ml of water. Four different mixtures were studied. These contained Soln. B (0.5ml) and Soln. A (0, 2, 5 or 10ml), together with buffer to make a total volume of 10.5ml. A similar series, with Soln. C in place of Soln. B, was also studied, and also a series in which the dopa, i.e. Soln. B, was replaced by buffer. The appropriate mixture (3ml) was placed in each of the spectrometer cells. Water (0.5ml) was added to the reference cell and H_2O_2 soln. (0.25ml) and enzyme soln. (0.25ml) were added to the sample cell. The absorption at 475nm was measured at 1min intervals for 20min.

(d) When the absorption spectrum of a reaction mixture containing dihydroxyfumaric acid was to be measured, a solution of dihydroxyfumaric acid (9.8mg) and L-tyrosine (4.7mg) in 0.2M-acetate buffer (10ml) was used. This solution (3ml) and H_2O_2 soln. (0.25ml) were placed in the sample cell. The reference cell contained buffer (3ml) and water (0.5ml). A solution of peroxidase (2mg) in water (0.25ml) was added to the sample cell, and the spectrum was scanned from 225 to 700nm.

Results and Discussion

The reaction mixtures of dopa, peroxidase and H_2O_2 showed absorption maxima at 332, 305, 418 and 475nm. The peak at 418nm was due to the peroxidase Compound III (Saunders *et al.*, 1964; Mason & Anan, 1960), an assignment that was confirmed by the presence of additional weak maxima at 545 and 580nm. The peaks at 305 and 475nm increased steadily, attaining maximum values after approx. 20min, and were assigned to dopachrome (Mason, 1948). The completion of the reaction after 20min

corresponded to the consumption of all the dopa, not to the exhaustion of the H_2O_2 , which would have been accompanied by a shift in the 418nm peroxidase peak to shorter wavelength. This shift, which was not observed in this case, was obtained in other experiments in which exhaustion of H_2O_2 occurred. The peak at 232nm was also present in a solution of peroxidase and H_2O_2 without added dopa.

The reaction mixture of tyrosine, peroxidase and H_2O_2 showed peaks at 240, 290 and 418nm. The intensities of the first two peaks increased with time, but had not reached a maximum after 30min; these peaks were assigned to 'dityrosine' [5,5'-bis-(2-amino-2-carboxyethyl)-2,2'-dihydroxybiphenyl]. The formation of such radical-coupling dimers from phenolic compounds by the action of peroxidase and H_2O_2 is well known (Westerfeld & Lowe, 1942; Saunders *et al.*, 1964); indeed Gross & Sizer (1959) have isolated 'dityramine' [5,5'-bis-(2-aminoethyl)-2,2'-dihydroxybiphenyl] from the products of oxidation of tyramine with peroxidase and hydrogen peroxide at pH9.2. The spectrum of dityramine is pH-dependent, and although Gross & Sizer (1959) did not record it at pH5.6, the spectrum that we observed at the latter pH value is close to their published spectrum obtained in more strongly acid solution. Moreover, di-*p*-cresol (5,5'-dimethyl-2,2'-dihydroxybiphenyl) showed absorption maxima at 288 and 328nm at pH5.6 and in alkaline solution respectively.

The reaction mixture containing dopa, tyrosine, peroxidase and H_2O_2 showed peaks at 240, 305, 418 and 475nm. The peaks at 305 and 475nm (corresponding to dopachrome) increased in intensity more rapidly than with dopa alone. After 10min the 475nm had reached a higher value than the maximum attained with dopa, but without tyrosine; subsequently the absorption at this wavelength increased only slowly. The 305nm peak underwent an apparent shift to shorter wavelength with time. After 45min it consisted of two overlapping maxima, one appearing as a shoulder on the more intense and shorter wavelength peak. This, together with the presence of the maximum at 240nm, was taken to be due to the formation of dityrosine. The 290nm (dityrosine) and the 305nm (dopachrome) peaks were superimposed, so that as dityrosine accumulated (initially at a lower rate than dopachrome) an apparent wavelength shift occurred.

Some of the experiments of Patel *et al.* (1971) were carried out in the presence of ascorbic acid to prevent oxidation of dopa. Our experiments confirmed that in the presence of ascorbic acid the first thing to happen was the oxidation of the latter, as seen by the disappearance of the 261nm maximum of this compound. When oxidation of ascorbic acid was complete, oxidation of tyrosine and dopa proceeded. The 475nm (dopachrome) peak was more intense in

tyrosine-dopa experiments, which had been carried out in the presence of ascorbic acid, than in those without ascorbic acid, although in experiments in which tyrosine was absent the presence of ascorbic acid made little difference. However, the E_{475} of the product from a mixture of tyrosine, ascorbic acid, peroxidase and H_2O_2 was correspondingly higher than that of the product obtained in the absence of ascorbic acid, although no maximum was present at 475 nm.

We also carried out experiments similar to those of Patel *et al.* (1974) in which the absorption at 475 nm was measured continuously for solutions containing different proportions of dopa and tyrosine, together with peroxidase and H_2O_2 . Our overall results were broadly similar to those given by Patel *et al.* (1974) in their Figs. 1-4.

The main differences noted in the reaction of dopa with (peroxidase+ H_2O_2) when tyrosine was also present were a greater initial rate of increase in E_{475} and a greater final extinction.

The greater rate of increase in E_{475} after a relatively short time might conceivably be the result of either (a) a higher concentration of dopa [as Patel *et al.* (1974) apparently believe], or (b) an acceleration of the reaction of dopa with (peroxidase+ H_2O_2) by tyrosine to yield dopachrome. Although, by the method used, it is not possible to measure accurately the rate of formation of dopachrome at zero time, it is nevertheless clear that the rate in the presence of tyrosine is higher than that in its absence, a fact that is inexplicable in terms of (a), but which is a natural consequence of (b). Bayse & Morrison (1971) noted this acceleration.

The greater final E_{475} of reaction mixtures of (dopa+peroxidase+ H_2O_2) in the presence of tyrosine (as opposed to in the absence of tyrosine) is attributed by Patel *et al.* (1974) to increased formation of dopachrome owing to hydroxylation of tyrosine. Bayse & Morrison (1971) had, however, concluded that no increase in E_{475} occurred in the presence of tyrosine. We therefore reinvestigated this experimental discrepancy and believe that the reason for its occurrence is that, whereas Patel *et al.* (1974) carried out their experiments at pH 5.6, Bayse & Morrison (1971) used pH 8. Dopachrome is subject to attack by nucleophiles and is less stable at pH 8 than at pH 5.6. Even at pH 5.6 we found that the maximum E_{475} attained increased marginally in the order 0.2M-acetate < 0.06M-acetate < 0.06M-phosphate. Most of our experiments were therefore carried out in the latter buffer solution, in which Mason (1948) measured the extinction coefficient of dopachrome. The experiments described by Patel *et al.* (1971) were carried out at 25°C, whereas those by Patel *et al.* (1974) were at 37°C. Our experiments were carried out at approx. 20°C, although we do not believe that within this range the temperature would alter the conclusions to

be drawn from the results. In fact we agree with Patel *et al.* (1974) that increased E_{475} is observed in the presence of tyrosine. Nevertheless we believe that all the results can be explained without postulation of the hydroxylation of tyrosine, and that the increase in E_{475} above that obtained from the original dopa is due to products of the reaction of tyrosine with (peroxidase+ H_2O_2) rather than to increased dopachrome formation.

According to our findings, for fixed concentrations of peroxidase and H_2O_2 , the intensity of the peak at 418 nm and of the associated shoulder absorption at 475 nm is increased in the presence of tyrosine. If the absorption at 475 nm for the reaction of (tyrosine+peroxidase+ H_2O_2) is subtracted from that of (tyrosine+dopa+peroxidase+ H_2O_2) after the same length of time (maximum E_{475} in the latter case), then the result is equal to that for (dopa+peroxidase+ H_2O_2) (maximum E_{475}). Moreover the E_{475} of the (dopa+tyrosine+peroxidase+ H_2O_2) solution continues to rise after that of the (dopa+peroxidase+ H_2O_2) solution has passed its maximum.

The curves shown in Figs. 1 and 2 were obtained by taking E_{475} values at intervals of 1 min of (i) a mixture of tyrosine, dopa, peroxidase and H_2O_2 , and (ii) the same solution with dopa absent, and subtracting the value of (ii) from the corresponding value of (i) at each point.

Our explanation of the 'biphasic absorption curve' of Patel *et al.* (1974) is that the initial steep portion represents (in the main) the rapid tyrosine-catalysed oxidation of the original dopa to dopachrome, and that the subsequent gently sloping portion represents

Fig. 1. Dopachrome formation from dopa/tyrosine mixtures in the presence of peroxidase and H_2O_2

The curves show the E_{475} (corrected for absorbance owing to oxidation products of dityrosine), after various reaction times, of mixtures of: (i) tyrosine (Soln. A; 2 ml) and dopa (Soln. B; 0.5 ml) in a total volume of 10.5 ml (Δ); (ii) as in (i), but with 5 ml of Soln. A ($\square$); (iii) as in (i), but with 10 ml of Soln. A ($\circ$); (iv) as in (i), but without tyrosine ($\blacktriangle$). Acetate buffer (0.2M) was used. Concentrations: dopa, 0.1 mM; H_2O_2 , 10 mM; tyrosine, (i) 0.4 mM, (ii) 1.05 mM, (iii) 2.1 mM.

Fig. 2. Dopachrome formation from dopa/tyrosine mixtures in the presence of peroxidase and H_2O_2 (with dopa concentration twice that of Fig. 1)

The procedure was exactly as in Fig. 1, but Soln. C was used in place of Soln. B, resulting in twice the dopa concentration (0.2 mM).

(in the main) absorption resulting from the products of the reaction of (tyrosine + peroxidase + H_2O_2). In Fig. 1 of Patel *et al.* (1974) the slope of Curve A declines just above the level of the maximum on curve B, as would thus be expected, and moreover curve A keeps on rising gently after curve B has reached its maximum.

Further, Patel *et al.* (1974) found that in experiments on the effect of varying the concentration of dopa, keeping that of tyrosine constant, the increase in E_{475} occasioned by tyrosine was independent of dopa concentration. If dopa is concerned in the hydroxylation of tyrosine, this result is surprising, whereas if the increase in E_{475} arises from tyrosine it is easily understood. As we would predict the increase in E_{475} was greater with increasing tyrosine concentrations and a constant concentration of dopa.

Following the observations made by Bayse & Morrison (1971) and Bayse *et al.* (1972), Patel *et al.* (1974) investigated the possible effect of the formation of dityrosine [tyrosine reacts quite rapidly with (peroxidase + H_2O_2) to yield dityrosine]. It is not clear why they used absorption at 315 nm to measure dityrosine, as in acid solution the maximum absorption of dityrosine occurs at 284 nm; in alkaline solution the maximum is at 316 nm (Gross & Sizer, 1959). Moreover, they stated that 'when 10^{-3} M-hydrogen peroxide was used.... differential dopachrome with tyrosine was noted, but was less than with 10^{-2} M.'. The levelling off of the curve in Fig. 5 of Patel *et al.* (1974) is no doubt the result of exhaustion of H_2O_2 and in the (tyrosine + dopa + peroxidase + 1 mM- H_2O_2) experiment the lower final E_{475} would be similarly explained.

Patel *et al.* (1974) also state that in alkaline solution peroxidative conversion of tyrosine into dopachrome is much more rapid, although no evidence is presented.

Patel *et al.* (1971) claimed qualitative evidence for

the formations of dopa by using the Arnow colour reaction. They carried out these reactions in the presence of ascorbic acid to prevent the formation of dopachrome, although Evans & Raper (1937) have stated that ascorbic acid interfered with the sodium molybdate colour reaction for dopa that they used. In the presence of neither dopa nor dihydroxy-fumaric acid were we able to show the formation of dopa from tyrosine by (peroxidase + H_2O_2) by using this colour reaction.

Patel *et al.* (1971) also used the method of Evans & Raper (1937) for separating dopa from tyrosine. When lead acetate is added to a weakly acidic solution of (tyrosine + dopa) and the pH of the mixture is adjusted to 8.5 by addition of aq. NH_3 , the dopa is precipitated as a lead salt, which can be filtered off. If the filtrate is then made more strongly alkaline, the lead salt of tyrosine is precipitated. The lead salts may be decomposed by H_2S to recover the free amino acids. Whereas Evans & Raper (1937) used various colour reactions etc. to measure the amount of tyrosine or dopa recovered, Patel *et al.* (1971) used u.v. spectra, which would not distinguish between tyrosine and dopa. We found that dityrosine appears to be precipitated as a lead salt at pH 8.5. Thus the product of reaction of (tyrosine + peroxidase + H_2O_2) in acetate buffer of pH 5.6, when treated with lead acetate and adjusted to pH 8.5 with aq. NH_3 , gave a precipitate. As dityrosine has strong absorption at 281 nm, this could account for the observed increase in E_{281} , which Patel *et al.* (1971) had attributed to newly formed dopa, and the observed disappearance of tyrosine is of course expected. Patel *et al.* (1971) had concluded that the yield of dopa was only 3%, although 29% of the tyrosine had been consumed. The low yield was attributed to the rapidity of conversion of dopa into further reaction products. The experiments were carried out in the presence of a reducing agent (ascorbic acid), so that the dopa should have been stable until all the reducing agent had been oxidized. The lead salt of ascorbic acid is also precipitated at pH 8.5.

The effect of tyrosine as an accelerator on the (dopa + peroxidase + H_2O_2) system reflects its function as a phenol, and this effect is not unknown. In terms of the theory of Yamazaki (1958), tyrosine would be regarded as an oxidogenic substrate. We therefore investigated the effect of a number of other phenolic compounds on the oxidation of dopa by (peroxidase + H_2O_2). *p*-Cresol produced a similar effect to tyrosine, although it was not expedient to use it in such high concentration as was possible with tyrosine, because the lower solubility in water of di-*p*-cresol led to precipitation. To produce the same accelerating effect as 3 mM-tyrosine it was necessary to add the following phenols at the approximate concentrations stated: 2,4,6-trimethylphenol (0.6 mM); di-*p*-cresol (0.2 mM); 3,4-dihydroxycinnamic acid (0.05 mM) (cf. Herzmann,

1957, 1959). These phenols resulted in no increase in absorption at 475nm, and phenylalanine had negligible effect on the rate of reaction and on the final E_{475} . It was impossible to study the effect of 2,4,6-tri-*t*-butylphenol because of its very low solubility in water.

We think that the effect of these phenolic compounds could be explained if (peroxidase + H_2O_2) reacts with a phenol to yield a phenoxy radical ($RO\cdot$), which can react rapidly with dopa to form a radical (dopa $\cdot$):

The compounds that are effective at very low concentrations are ones from which the phenoxy radical is expected to be relatively stable. Thus the added phenolic compound has to be capable of reacting with (peroxidase + H_2O_2) to yield a radical, which cannot itself undergo further rapid reaction to yield other products, but which passes on its radical character to dopa. The resulting dopa radical undergoes an irreversible reaction leading to dopachrome.

Patel *et al.* (1971, 1974) claimed evidence for the formation of dopa in the system (tyrosine + dihydroxyfumaric acid + peroxidase + H_2O_2) by (a) the use of the Arnow colour reaction, (b) isolation of dopa as the lead salt (the reaction having been carried out in the presence of sodium ascorbate to prevent oxidation of dopa to dopachrome) and (c) observation of increased E_{475} in the absence of reducing agent. Although the authenticity of the sample of dihydroxyfumaric acid that we used was checked by melting point and by its u.v. spectrum in both water and ether, nevertheless we failed to obtain a positive result by the use of the Arnow test when we attempted to repeat the above work. The use of the lead salt here is subject to the same criticism as in the (tyrosine + dopa) case above.

We measured E_{475} at various times of a solution containing tyrosine, dihydroxyfumaric acid, peroxidase and H_2O_2 . This maintained a steady, low value for 4 min, then rose rapidly during a period of 1 min; the rate of increase then slowed down, and a maximum absorption was reached after 14 min (Fig. 3). When further H_2O_2 was added, the absorption increased again.

The spectrum of this reaction mixture was therefore scanned at various times. The initial, steady absorption at 475nm was evidently due to the enzyme. The first 4 min involved the complete oxidation of the dihydroxyfumaric acid, as shown by the disappearance of the 296nm peak. In a control experiment, in which dihydroxyfumaric acid was absent, oxidation of tyrosine began immediately. The comparatively low maximum in the presence of dihydroxyfumaric acid appears to be due to exhaustion of H_2O_2 , some of which is used in the oxidation of the dihydroxyfumaric acid.

Fig. 3. E_{475} of a solution of tyrosine containing dihydroxyfumaric acid, undergoing oxidation by peroxidase in the presence of H_2O_2

(□) A solution was used containing tyrosine, dihydroxyfumaric acid, peroxidase and H_2O_2 as detailed in paragraph (d) of the Experimental section. (○) As above, but with dihydroxyfumaric acid omitted. Acetate buffer (0.2M) was used.

It seems that after oxidation of dihydroxyfumaric acid is complete the rate of oxidation of tyrosine is greater than when dihydroxyfumaric acid was initially absent, i.e. that the oxidation product of dihydroxyfumaric acid slightly accelerates the oxidation of tyrosine, probably by an effect like that produced by phenols (dioxosuccinic acid exists in aqueous solution in a hydrated form, i.e. as dihydroxytartaric acid). Dityrosine itself is virtually free from absorption at 475nm, so the absorption at that wavelength produced in a solution of tyrosine oxidized by (peroxidase + H_2O_2) must be due to some product of interaction of dityrosine with the enzyme, to some by-product of the oxidation of tyrosine, or to some product of the further oxidation of dityrosine. The curves suggest that in fact oxidation of tyrosine to dityrosine proceeds even in the presence of dihydroxyfumaric acid (horizontal portion of curve), and that as soon as the oxidation of dihydroxyfumaric acid is completed, there is a very rapid increase in absorption because of, e.g. the further oxidation of the dityrosine already formed (very steep portion of curve). As soon as the latter process is completed, the less steep slope follows, although presumably this slope represents the rate of oxidation of tyrosine since the beginning. In agreement with this interpretation we found that when dihydroxyfumaric acid (in amount greater than that capable of being oxidized

by the peroxidase and H_2O_2 present) was added to a solution of tyrosine which had been oxidized by (peroxidase + H_2O_2) the absorption at 475 nm diminished.

Thus we have been unable to find any evidence of the formation of dopa in this system, and even if this happened, its significance *in vivo* would be doubtful. Non-specific hydroxylation of aromatic compounds by oxygen in the presence of peroxidase and dihydroxyfumaric acid is well known (Mason *et al.*, 1957a), the introduced oxygen atom arising from molecular oxygen (Mason *et al.*, 1957b). The mechanism here involved is probably quite different from that in the absence of dihydroxyfumaric acid. The peroxidase produces a radical from the dihydroxyfumaric acid, which is able to reduce oxygen (Ullrich *et al.*, 1965). Ascorbic acid cannot replace dihydroxyfumaric acid.

A mixture of tyrosine, dihydroxyfumaric acid and peroxidase was oxygenated vigorously for 30 min. The precipitate was then removed, and the spectrum of the filtrate showed that the dihydroxyfumaric acid had been almost completely removed. When the filtrate was treated with H_2O_2 , rapid build-up of an absorption maximum at 475 nm occurred, part of which could be attributed to oxidation products of dihydroxytyrosine, but which was mainly due to dopachrome. Thus hydroxylation of tyrosine can be brought about by peroxidase under conditions of vigorous oxygenation; H_2O_2 is not required (cf. Buhler & Mason, 1961).

Bayse & Morrison (1971) pointed out that several alternative interpretations of the experimental results of Okun, Edelstein and co-workers (Okun *et al.*, 1970) are possible. Among these, they stated 'There is some experimental evidence for the involvement of peroxide in the later steps of melanin formation', and cited a paper by Swan & Wright (1954). We take exception to this statement. It is true that Swan & Wright (1954) demonstrated the involvement of H_2O_2 in melanogenesis, but only in a side reaction, involving oxidative fission of indole rings already part of the polymer; we have no evidence that H_2O_2 was involved in the oxidation of tyrosine or dopa to dopachrome, and hence by way of 5,6-dihydroxyindole to the melanin polymer.

In summary, although we were able to reproduce a considerable part of the experimental results of Patel *et al.* (1971, 1974) (although not their results involving dihydroxyfumaric acid), we nevertheless do not believe that these results can be taken as evidence that tyrosine is converted into dopa by (horseradish peroxidase + H_2O_2) in the presence of either dopa or dihydroxyfumaric acid *in vitro*. If such conversion does indeed occur, it should be possible to establish this by a suitable tracer experiment. The ability of peroxidase to oxidize dopa is not, however, in doubt.

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