Tyrosinase Activity in the Pigmented Cells of the Nucleus Substantiae Nigrae

II. Further Observations on Monophenolase Activity

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With one plate (fig. 1)

SUMMARY

In a previous investigation of tyrosinase activity in the cells of the nucleus substantiae nigrae of adult cats and monkeys, diphenolase, but not monophenolase activity, was detected. This is possibly due to inhibition of monophenolase. When the copper radicle in tyrosinase is oxidized, monophenolase cannot be detected, but reducing agents activate and unmask this component of tyrosinase.

The effects of reducing agents in activating monophenolase have been investigated. Tyrosine is converted to melanin by monophenolase in adult nigra cells of the cat and monkey when the enzyme is activated by the reducing agents L-dopa, L-ascorbic acid, and L-adrenaline. Variations in physical factors influence the intensity of the activated monophenolase reaction resulting from incubation of sections in tyrosine-dopa mixtures; the reaction is inhibited by general enzyme inhibitors and specific tyrosinase inhibitors.

The significance of the results in relation to tyrosinase activity in adult nigra cells is discussed.

INTRODUCTION

THE pigmented cells of the nucleus substantiae nigrae of adult cats and monkeys convert 3,4-dihydroxyphenylalanine (dopa) to melanin pigment *in vitro*, and evidence has been presented to show that this is due to the presence of the diphenolase activity of the enzyme tyrosinase in the cells (Marsden, 1960). Monophenolase activity of tyrosinase, however, cannot be detected and adult nigra cells are apparently incapable of oxidizing tyrosine to dopa, which is the first step in melanin formation. In order to show that the pigment in nigra cells is formed by the action of tyrosinase on the amino acid tyrosine, the metabolic system responsible for the production of melanin in other pigmented tissues in the mammalian body (Fitzpatrick, Brunet, and Kukita, 1958; Fitzpatrick and Kukita, 1959), it is necessary to demonstrate the presence not only of diphenolase activity, but also of the monophenolase activity of tyrosinase.

The oxidation of tyrosine to dopa by monophenolase activity is slow in onset, increasing in rapidity after an initial time-lag or induction period (Lerner and Fitzpatrick, 1950). The duration of the induction period is dependent on the redox potential of the system; reducing agents shorten the period, by lowering the redox potential, while conversely, oxidizing agents increase the time lag (Figge, 1948). No similar induction period exists in the

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oxidation of dopa to dopa-quinone by diphenolase activity. The oxygen tracer studies of Mason, Fowlks, and Peterson (1955) have shed new light on the mechanism of tyrosinase action and the method whereby reducing agents shorten the induction period in tyrosine oxidation. Mason (1956) has suggested that the copper radicle of tyrosinase may exist in either the reduced or oxidized state. In life, tyrosinase may exist as a reduced enzyme active towards both tyrosine and dopa, or, because of a high prevailing redox potential, as an oxidized form, which is inert but capable of activation towards tyrosine, while remaining readily active towards dopa. In the presence of reducing agents, two cupric atoms in tyrosinase are reduced to cuprous atoms, and the enzyme becomes active towards tyrosine. This mechanism may be invoked to explain the apparent absence of monophenolase activity in adult pigmented nigra cells; the tyrosinase therein perhaps being in the oxidized state.

The most effective agent for shortening the induction period in tyrosine oxidation is dopa, which, in addition to serving as a substrate, can itself function as an activator by reducing cupric tyrosinase to cuprous tyrosinase, with the consequent unmasking of monophenolase activity (Lerner, Fitzpatrick, Calkins, and Summerson, 1949). Tyrosine oxidation by tyrosinase is therefore an autocatalytic process. Ascorbic acid (Arman and Jones, 1949) and substances structurally related to dopa, such as adrenaline (Lerner, Fitzpatrick, and Summerson, 1949) also shorten the induction period by their reducing action, but not nearly so effectively as dopa. The possibility that reducing agents might unmask monophenolase activity has been investigated in the present study.

Materials and Methods

As in the first part of these investigations, frozen sections were obtained from the mid-brain of adult cats and monkeys, previously fixed for 3 h in 10% neutral formalin (Marsden, 1960). Sections were incubated for 24 to 48 h at 37° C in a solution containing 1 : 1000 L-tyrosine in 0·1 M phosphate buffer at pH 6·8, to which were added potential activators of monophenolase activity. The reducing agents, L-dopa, L-ascorbic acid, L-adrenaline, and sodium hydrosulphite were separately added to the incubating solution at concentrations of 0·005%. Sections incubated in L-tyrosine alone or in the same concentrations of the individual reducing agents alone were used as controls. Sections were also incubated in buffer solution.

The addition of L-dopa to the incubating solution of L-tyrosine was most likely to activate monophenolase activity, if tyrosinase was present in the oxidized state in adult nigra cells. The existence of monophenolase activity could only be demonstrated by showing that the tyrosine was converted to melanin. It was, of course, possible that, in a tyrosine-dopa mixture, the dopa was acting as a substrate for tyrosinase, resulting in blackening of nigra cells by the melanin produced by diphenolase, rather than by monophenolase activity. To eliminate this possibility, sections were incubated in radioactive L-tyrosine C^{14}, to which non-radioactive L-dopa was added, and were subsequently

subjected to autoradiography, according to the method of Kukita and Fitzpatrick (1955). Control sections were incubated in this solution with the addition of 0·01 M sodium diethyldithiocarbamate, a copper-binding inhibitor of tyrosinase; sections were also incubated in L-tyrosine C^{14} alone.

Experiments were conducted to determine the nature of the reaction resulting from incubation of sections in tyrosine-dopa mixtures. The effects of variations of physical factors on the reaction were investigated and inhibitors of melanin formation were employed. Sections were incubated in buffered L-tyrosine, to which L-dopa was added, and the pH concentration of L-tyrosine, the duration of incubation, and the temperature were independently varied as in the previous investigation (Marsden, 1960). Sections were incubated for 24 h at 37° C in 1:1000 L-tyrosine in phosphate buffer at pH 6·8, to which the following inhibitors of melanin formation were added separately: hydrogen sulphide, sulphur dioxide, potassium cyanide, sodium azide, phenylurethrane, 'tween 20', hydroquinone, sodium diethyldithiocarbamate, thiourea, phenylthiourea, α-naphthylthiourea, and 4-chlororesorcinol. The modes of action of these inhibitors and the concentrations employed were discussed in the previous investigation (Marsden, 1960).

Results

After incubation of mid-brain sections of adult cat and monkey in L-tyrosine, to which L-dopa had been added, the cells of the nucleus substantiae nigrae were blackened (fig. 1, A). No such blackening occurred in control sections (fig. 1, B). This blackening resembled that observed after incubation of sections in L-dopa alone, and was due to the presence of many granules of black melanin pigment in the cytoplasm of the nigra cells. Although a number of isolated cells in the reticular formation of the mid-brain were also blackened, the cells of other nuclear groups were free of pigment. Incubation of sections in L-tyrosine, to which ascorbic acid (0·005%) or L-adrenaline (0·005%) had been added, produced a similar result, although the blackening of nigra cells was less intense than when dopa was used as the activator (fig. 1, C). No blackening of nigra cells followed incubation of sections in L-tyrosine with sodium hydrosulphite (0·005%). In sections incubated in L-dopa (0·005%) or L-adrenaline (0·005%) alone the nigra cells were darker than in control sections incubated in buffer alone, but the intensity of the reaction was slight, and the number of darkened cells was smaller than in sections incubated in tyrosine-dopa or tyrosine-adrenaline mixtures. No change could be detected between sections incubated in L-ascorbic (0·005%) or sodium hydrosulphite (0·005%) alone and similar material incubated in buffer solution alone.

In the autoradiographs, concentrations of silver grains were deposited over the blackened nigra cells (fig. 1, D, E); but no such deposition of silver occurred in other cells in the same section, or in the control sections (fig. 1, F).

The variations of pH, concentration of L-tyrosine, duration of incubation, and temperature all affected the intensity of blackening of nigra cells which resulted from incubation of sections in tyrosine-dopa mixtures. The reaction

occurred only between pH 6·8 and 7·4; its intensity increased with greater concentrations of L-tyrosine or with increased periods of incubation; no reaction took place at 4° C, or at 60° C, but specific blackening occurred after 72 h of incubation at 18° C, or 24 h of incubation at 37° C. The blackening of nigra cells resulting from incubation of sections in tyrosine-dopa mixtures was completely suppressed by the majority of inhibitors employed, namely hydrogen sulphide, sulphur dioxide, potassium cyanide, 'tween 20', hydroquinone, sodium diethyldithiocarbamate, thiourea, phenylthiourea, α-naphthylthiourea, and 4-chlororesorcinol, but sodium azide and phenylurethrane did not prevent the reaction.

Discussion

The monophenolase activity of tyrosinase could not be detected in the adult nigra cells of the cat and monkey, although diphenolase activity was present (Marsden, 1960). Dopa, but not tyrosine, was converted to melanin by nigra cells. However, it has been found possible to promote the conversion of tyrosine to melanin in nigra cells by the use of reducing agents in the reaction. Blackening of nigra cells took place when mid-brain sections were incubated in L-tyrosine, to which reducing agents such as L-dopa, L-ascorbic acid, or L-adrenaline were added. The apparent failure of sodium hydrosulphite in this respect is inexplicable. By far the most effective of these agents was L-dopa, which might itself have acted as a substrate for the diphenolase activity of tyrosinase. The existence of monophenolase activity in nigra cells could only be demonstrated by showing that tyrosine was converted to melanin therein.

Certain evidence adduced from the experiments conducted in this study confirmed that the nigra cells possessed the capacity to promote the conversion of tyrosine to melanin, in the presence of reducing agents. The deposition of silver in autoradiographs prepared after incubation of sections in radioactive L-tyrosine C^{14}, to which non-radioactive dopa had been added, must have been due to the uptake of the labelled amino-acid. Tyrosine is water-soluble, and, during processing, the sections were subjected to a period of 8 h of washing to remove all free tyrosine. The silver deposition was observed over the nigra cells, which were blackened with melanin; this indicated the presence of radioactivity in them. The radioactivity could only have been due to the

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Fig. 1 (plate). A, the nucleus substantiae nigrae of the adult monkey after incubation in L-tyrosine, with the addition of L-dopa.

B, the nucleus substantiae nigrae of the adult monkey after incubation in L-tyrosine alone.

C, the nucleus substantiae nigrae of the adult monkey after incubation in L-tyrosine, with the addition of L-ascorbic acid.

D, a cell of the nucleus substantiae nigrae of the adult monkey after incubation in radioactive L-tyrosine C^{14}, with the addition of L-dopa. Cell in focus.

E, the same cell as in D. The overlying photographic emulsion in focus. Silver granules are concentrated over the nigra cell.

F, the nucleus substantiae nigrae of the adult monkey after incubation in radioactive L-tyrosine C^{14} alone. No silver grains are visible over the nigra cells. Haematoxylin and eosine.

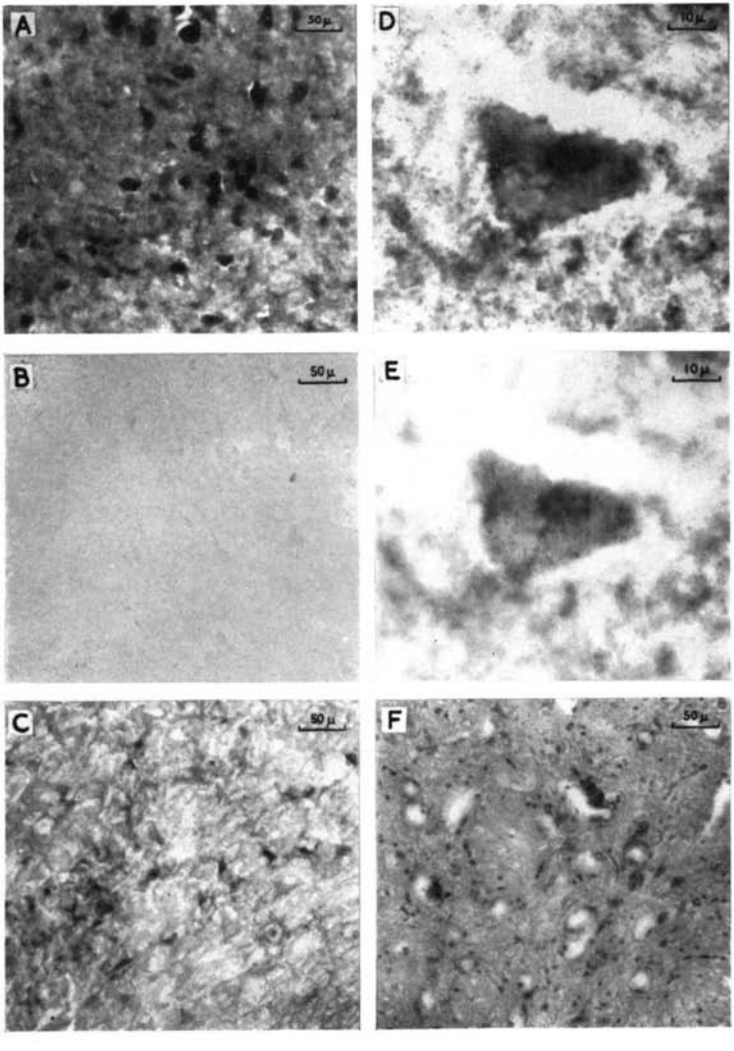

FIG. 1

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conversion of the labelled L-tyrosine to melanin, which, being insoluble in water, remained after washing. The absence of radioactivity in control sections indicated that the period of washing was effective in removing all free L-tyrosine C^{14}. In addition, the inhibitors 'tween 20' and hydroquinone prevented blackening of nigra cells incubated in tyrosine and dopa, but did not interfere with blackening of nigra cells when incubated in dopa alone (Marsden, 1960). These two substances have been shown to prevent the conversion of tyrosine to dopa, but not the subsequent oxidation of dopa to melanin (Lerner and Fitzpatrick, 1950; Denton, Lerner, and Fitzpatrick, 1952).

These findings indicated that tyrosine was converted to melanin in the nigra cells of the adult cat and monkey, when reducing agents were present. The factor responsible for this reaction was probably the monophenolase component of tyrosinase, and the actions of the remaining inhibitors confirmed this conclusion. The general enzyme inhibitors—hydrogen sulphide, sulphur dioxide, and potassium cyanide—prevented the reaction. The cytochrome oxidase inhibitors, sodium azide, and phenylurethrane (Pearse, 1953), did not prevent blackening of nigra cells after incubation in tyrosine and dopa, and this indicated that this group of enzymes was not responsible for the reaction. However, specific inhibitors of tyrosinase prevented the conversion of tyrosine to melanin by nigra cells. The modes of action of these inhibitors were discussed in the previous investigation (Marsden, 1960). These results suggested that tyrosinase was present in the oxidized state in adult nigra cells, and hence required activation by reducing agents to demonstrate its monophenolase activity.

Recently Shimizu, Matsunami, and Onishi (1960) demonstrated high concentrations of ascorbic acid in the nucleus locus coeruleus, the dorsal motor nucleus of the vagus, and the area postrema. Pigment similar to that found in the nucleus substantiae nigrae is present in these sites (Olszewski and Baxter, 1954) and may reasonably be presumed to be formed by the action of tyrosinase on tyrosine. The presence of ascorbic acid in these particular nerve-cells provides an activator of monophenolase activity, and thus pigment formation from tyrosine could take place at these sites.

No function can be ascribed to pigment in the cells of the nucleus substantiae nigrae or in other nerve-cells. It is possible that melanin in the brain is a by-product of some other essential metabolic process occurring in pigmented cells. Tyrosine is the precursor, not only of melanin, but also of the catechol amine dopamine, which has been demonstrated in the brains of man and animals, especially in the basal ganglia (Carlsson, Lindqvist, Magnusson, and Waldeck, 1958; Bertler and Rosengren, 1959*a*), and, ultimately of noradrenaline and adrenaline (Lerner, 1953), which have also been shown to be present in nervous tissue (Vogt, 1954). Bertler and Rosengren (1959*b*) have concluded that dopamine has a specific function in the brain, concerned with motor activity, while Vogt (1954) suggested that noradrenaline and adrenaline are concerned with sympathetic activities in the brain. Recently Bertler (1960) has

shown the presence of large amounts of dopamine in the human substantia nigra, a nucleus recognized as an integral part of the extrapyramidal motor system. The first step in the synthesis of dopamine, noradrenaline, or adrenaline is the oxidation of tyrosine to dopa, and the present investigation has demonstrated the presence of the enzyme responsible for this reaction, tyrosinase, in the pigmented cells of the nucleus substantiae nigrae of the adult cat and monkey.

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