

Tyrosinase Activity in the Pigmented Cells of the Nucleus Substantiae Nigrae. I. Monophenolase and Diphenolase Activity

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With one plate (fig. 1)

SUMMARY

The method of formation of black pigment in cells of the nucleus substantiae nigrae has been investigated by employing histochemical techniques which detect tyrosinase, the enzyme responsible for melanin formation in skin melanocytes. Frozen sections of the mid-brains of adult monkeys and cats were incubated in L-tyrosine, to detect the monophenolase activity of tyrosinase; other sections were incubated in L-dopa to detect the diphenolase (dopa oxidase) activity of the enzyme. The dopa, but not the tyrosine, was converted into melanin by the cells of the nucleus substantiae nigrae, resulting in blackening of the cytoplasm.

The intensity of blackening of nigra cells resulting from incubation in L-dopa depended on physical factors. The optimum pH of the reaction ranged between 6.8 and 7.4; the optimum temperature was 37° C; the concentration of substrate employed and the duration of incubation were additional factors which influenced the intensity of the reaction. The conversion of dopa to melanin by nigra cells was prevented by general enzyme inhibitors and by specific inhibitors of tyrosinase, which confirmed the specificity of the reaction for the diphenolase activity of tyrosinase.

The significance of the presence of diphenolase activity, in the absence of monophenolase activity, in adult nigra cells has been discussed in relation to pigment formation in the brain.

INTRODUCTION

PIGMENT granules are present in the cells of certain nuclei of the adult human brain-stem, especially in the nucleus substantiae nigrae and the nucleus locus coeruleus. They have also been demonstrated in the nucleus substantiae nigrae of many mammals (Marsden, 1960). The significance of this pigment in the brain and the biochemical processes involved in its formation are unknown. The brown or black intracytoplasmic granules are usually considered to be melanin, and resemble melanin granules in skin melanocytes in many of their physical and chemical properties (Lillie, 1954). Pigment in the brain may be formed by processes similar to those producing melanin in the skin melanocytes. The chemical reactions occurring in the formation of melanin in the skin were first established by Raper (1928) and have subsequently been confirmed (Lerner, 1953).

Cutaneous melanin is formed by the catalytic action of tyrosinase, an

enzyme present in melanocytes, on the amino-acid tyrosine. Tyrosinase catalyses the oxidation of tyrosine to 3,4-dihydroxyphenylalanine (dopa), and the subsequent oxidation of dopa to dopa-quinone. Dopa-quinone is subsequently converted through intermediate stages to 5,6-dihydroxyindole, which finally polymerizes to form melanin. Tyrosinase, which is a member of the copper-containing group of oxidases termed phenolases (Mason, 1956), exhibits a monophenolase and a diphenolase activity. The monophenolase activity catalyses the oxidation of tyrosine to dopa, and the diphenolase (dopa oxidase) activity catalyses the oxidation of dopa to dopa-quinone (Lerner and Fitzpatrick, 1950), which is subsequently converted to melanin non-enzymatically. Both of these activities of the one enzyme are essential in the production of cutaneous melanin.

The monophenolase activity of tyrosinase in the skin may be detected histochemically by incubating fresh tissue in L-tyrosine. In the presence of monophenolase activity the tyrosine is converted into melanin with blackening of the cytoplasm of cells containing the enzyme (Fitzpatrick, Becker, Lerner, and Montgomery, 1950). The diphenolase activity of tyrosinase may be detected by using dopa as the substrate; this results in a similar blackening of the cytoplasm of cells containing the enzyme, owing to the formation of melanin from dopa (Bloch, 1916). The similarity of pigment in the brain-stem to cutaneous melanin has suggested that it may also be formed by the action of tyrosinase on tyrosine, and therefore the histochemical techniques for demonstrating the two activities of the enzyme in the skin have been applied to the pigmented cells of the nucleus substantiae nigrae.

MATERIAL AND METHODS

The monkey and the cat both possess pigmented nigra cells (Scherer, 1939; Brown, 1943) and were used for this study. Under conventional light microscopy, such as was used in this study, the pigment was not visible in unstained preparations. Therefore additional pigment formed as a result of monophenolase or diphenolase activity would be immediately apparent. The brains of 4 adult Rhesus monkeys (*Macaca rhesus*) and 5 adult cats (*Felis felis*) were removed immediately after death, and fixed in 10% neutral formalin for 1 h. The mid-brain was then removed and fixed for a further 2 h in the same solution. Frozen transverse sections of the mid-brain, 10 to 15 μ thick, were mounted on clean glass slides and dried in air to ensure adhesion. These sections were either used immediately or stored in a deep-freeze at -20° C for up to 3 weeks.

Reaction for monophenolase activity

A modification of the method of Fitzpatrick, Becker, Lerner, and Montgomery (1950) was employed. Sections were incubated for 24, 36, and 48 h at 37° C in 1:1000 L-tyrosine in 0.1 M phosphate buffer at pH 6.8. Sections incubated in buffer solutions alone were used as controls.

Reaction for diphenolase activity

A modification of the method of Bloch (1916) was employed. Sections were incubated for 24 h at 37° C in 1:1000 L-dopa in 0.1 M phosphate buffer at pH 6.8. Sections incubated in buffer solutions alone were used as controls.

The effects of variations of the following physical factors on the reaction for diphenolase activity were independently investigated:

pH. Sections were incubated for 24 h at 37° C in 1:1000 L-dopa buffered at pH 5.0, 6.0, 6.8, 7.4, and 8.0.

Substrate concentration. Sections were incubated for 24 h at 37° C in 1:1000, 2:1000, and 3:1000 concentrations of L-dopa buffered at pH 6.8.

Duration of incubation. Sections were incubated for 2, 6, 12, 18, 24, 48, and 56 h at 37° C in 1:1000 L-dopa buffered at pH 6.8.

Temperature. Sections were incubated for 24 h at 4° C, 18° C, 37° C, and 60° C in 1:1000 L-dopa buffered at pH 6.8.

The specificity of the diphenolase reaction was investigated by using a series of inhibitors of melanin formation. Lerner and Fitzpatrick (1950) and Lorincz (1954) described a number of groups of inhibitors of cutaneous melanogenesis on the basis of their modes of action. Representatives of these were individually added to the buffered L-dopa solution used in the concentrations detailed below, and sections were incubated as before for 24 h at 37° C:

General enzyme inhibitors. Hydrogen sulphide (saturated solution), sulphur dioxide (saturated solution), potassium cyanide (1% and 0.1%).

Cytochrome oxidase inhibitors. Sodium azide (0.001 M), phenylurethane (0.003 M).

Substances which prevent the oxidation of tyrosine to dopa. 'Tween 20' (10% solution), hydroquinone (0.07 M).

Copper-binding tyrosinase inhibitors. Sodium diethyldithiocarbamate (0.01 M), thiourea (1 M), phenylthiourea (0.01 M), α -naphthylthiourea (0.01 M).

Competitive inhibitor of tyrosinase. 4-chlororesorcinol (0.01 M).

RESULTS

Monophenolase activity in nigra cells

The method used to demonstrate monophenolase activity resulted in a negative response. No blackening of the cytoplasm was detectable in the nigra cells of the animals employed after incubation in L-tyrosine.

Diphenolase activity in nigra cells

The method used to demonstrate diphenolase activity resulted in a positive response, consisting of intense blackening of the nigra cells in both animals (fig. 1, A). No blackening occurred in the nigra cells in sections incubated in buffer solution alone (fig. 1, B). The blackening of nigra cells was due to the presence of varying concentrations of small black granules of pigment, produced

by the conversion of dopa to melanin. Small numbers of isolated pigment granules were also scattered uniformly throughout the sections, but pigment was most heavily concentrated specifically in the nigra cells. A number of isolated cells in the reticular formation of the mid-brain were also blackened, whereas the cells of other nuclear groups were almost indistinguishable from the surrounding background (fig. 1, C). In the less intensely blackened nigra cells, the pigment granules were separate (fig. 1, D), while in heavily blackened cells they were concentrated into a dense, black mass (fig. 1, E). Pigment granules were confined to the cytoplasm of nigra cells and were not found in the nucleus, nor did they extend into the cell processes (fig. 1, F). In some cells granules were concentrated around the nucleus, producing a darkly staining 'perinuclear ring' (fig. 1, G). Granules were also concentrated in the tissue in the immediate vicinity of the nigra cells.

Variations in physical factors influenced the intensity of the reaction. Incubation at a pH below 6.8 produced no blackening of nigra cells, but at pH 6.8 to 7.4 the black cells stood out distinctly from the pale background. The intensity of the reaction was not greatly increased by incubation at a pH above 7.4, but the scattered background pigmentation was markedly intensified. Incubation of sections in different concentrations of L-dopa showed that the higher the concentration of substrate employed, the greater was the intensity of blackening of nigra cells. Within limits, the longer the period of incubation the greater was the intensity of the result, the maximum blackening of nigra cells occurring after 24 h incubation. Even after 2 h a few cells had blackened selectively in the substantia nigra. The maximum intensity of the blackening of nigra cells occurred when sections were incubated at 37° C. No reaction took place when sections were incubated at 4° C, and sections incubated at 18° C took up to 72 h to exhibit blackening of nigra cells; after incubation at 60° C the whole section darkened, but there was no specific blackening of nigra cells.

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

B, the nucleus substantiae nigrae of the adult monkey after incubation in buffer solution alone.

C, a cell from the nucleus ruber of the adult monkey after incubation in L-dopa. The cytoplasm is pale and free of pigment.

D, a cell from the nucleus substantiae nigrae of the adult monkey after incubation in L-dopa. The cytoplasm of this lightly blackened cell is packed with black pigment granules.

E, a cell from the nucleus substantiae nigrae of the adult monkey after incubation in L-dopa. The cytoplasm of this heavily blackened cell is completely obscured by a dense mass of black pigment.

F, a cell from the nucleus substantiae nigrae of the adult monkey after incubation in L-dopa. Pigment is concentrated within the cytoplasm of the cell, and granules lie in the surrounding tissue. The cell nucleus and nerve processes are free of pigment.

G, a cell from the nucleus substantiae nigrae of the adult monkey after incubation in L-dopa. Pigment is concentrated in a 'perinuclear ring'.

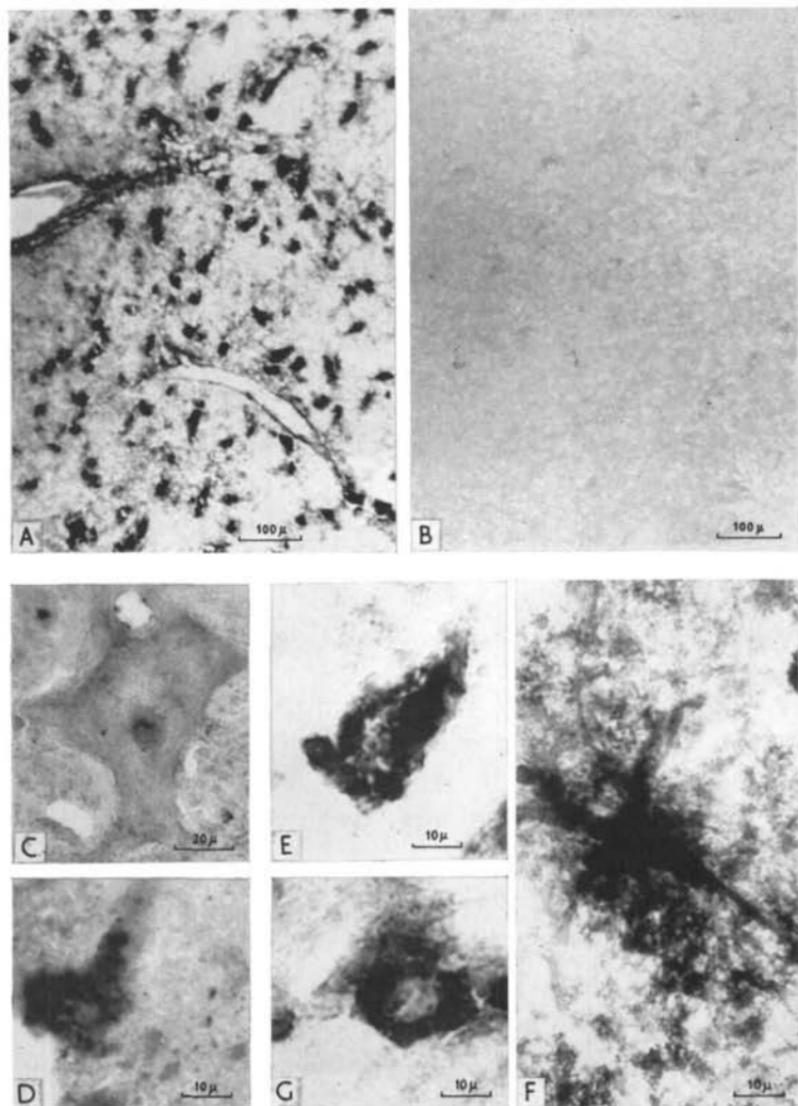


FIG. 1

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Of the inhibitors employed, the majority, namely hydrogen sulphide, sulphur dioxide, potassium cyanide, sodium diethyldithiocarbamate, thiourea, phenylthiourea, α -naphthylthiourea, and 4-chlororesorcinol, prevented the conversion of dopa to melanin and the subsequent blackening of nigra cells. Sections incubated in L-dopa, with the addition of these inhibitors individually, showed no differences from similar sections incubated in buffer solutions alone. Blackening of nigra cells still occurred when sections were incubated in the presence of sodium azide, phenylurethane, 'tween 20', and hydroquinone.

DISCUSSION

The negative results obtained with the reaction for the monophenolase activity of tyrosinase could be interpreted as indicating the absence of this enzyme in adult nigra cells. However, the positive result obtained with the reaction for the diphenolase activity of tyrosinase excludes this possibility. The specificity of this latter reaction for diphenolase activity has been confirmed by studying the effects of variations of physical factors and by the use of inhibitors of tyrosinase.

Although the oxidation of dopa to melanin can occur in the absence of an enzyme in solutions above pH 7.0 (Lerner, Fitzpatrick, Calkins, and Summerson, 1949), a positive diphenolase reaction in nigra cells was obtained at pH 6.8. The cytochrome oxidase group of enzymes can also oxidize dopa to melanin (Herrman and Boss, 1945), but the addition of two inhibitors of cytochrome oxidase activity, sodium azide and phenylurethane (Pearse, 1953), did not interfere with the positive diphenolase reaction in nigra cells. Finally, the diphenolase reaction was completely suppressed by specific inhibitors of tyrosinase. The copper-binding inhibitors employed acted by forming weakly dissociable complexes with the copper moiety of the enzyme (Lerner and Fitzpatrick, 1950), the presence of copper ions being essential for tyrosinase action (Lerner, Fitzpatrick, Calkins, and Summerson, 1950), while the competitive inhibitor acted as a competitive substrate for the enzyme (Fitzpatrick and Kukita, 1959).

The absence of monophenolase activity in the presence of undoubted diphenolase activity in adult nigra cells is puzzling. In this respect these pigmented cells resemble certain pigmented melanocytes in the skin and other tissues. Monophenolase activity cannot be detected in skin melanocytes unless pigment formation is occurring, nor in the melanocytes of human grey hair, even though these cells retain the ability to oxidize dopa (Fitzpatrick, Brunet, and Kukita, 1958). It is probable that monophenolase and diphenolase activity can be detected together only in melanocytes which are in the process of active pigment formation. The failure to detect monophenolase activity in adult nigra cells may indicate that pigment formation has temporarily or permanently ceased. However, the demonstration of diphenolase activity in the nigra cells of two animals that possess pigmented cells in the nucleus substantiae nigrae suggests that tyrosinase plays an important role in the formation of the pigment, which is probably melanin derived from tyrosine.

My thanks are due to Professor D. V. Davies for his encouragement and help in this work; to Dr. W. Hewitt for his criticism of the manuscript; to Mr. D. N. Petts for technical assistance; and to Mr. J. S. Fenton for the photomicrographs.

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